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VOLUME 25 NUMBER 4

STAIN TECHNOLOGY

A JOURNAL FOR MICROTECHNIC

OFFICIAL ORGAN OF BIOLOGICAL STAIN COMMISSION

OCTOBER 1950

CONTENTS

ORIGINAL ARTICLES

The Mechanism of the Gram Reaction. II. The Function of Iodine in the Gram Stain. TOD MITTWER, JAMES W. BARTHOLOMEW, and BURTON J. KALLMAN..... 169

Dye Exchange in Bacterial Cells, and the Theory of Staining. J. W. BARTHOLOMEW, M. A. ROBERTS, and E. EDWARD EVANS..... 181

A Simple Histochemical Reaction for Aldehydes. J. P. ARZAC..... 187

The New Domestic Cresyl Echt Violet. THERESA M. BANNY and GEORGE CLARK..... 195

A Chrome-Alum Preparation for Delicate and Difficult Fixations. FRANK AMMERMAN 197

Spectrophotometric Characteristics and Assay of Biological Stains. III. The Xanthenes. ARTHUR J. EMERY, JR., FRANCES KNAPP HAZEN, and ELMER STOTZ..... 201

The Corneal Epithelium as a Source of Mammalian Somatic Mitoses. HELEN GAY and BERWIND P. KAUFMANN..... 209

Foot Focusing Device for Binocular Dissecting Microscope. GEORGE H. MICKEY.... 217

Mounting Large Series of Single Sections on Few Slides. OCTAVIA HALL..... 219

Decreasing the Time Required for Making an Alizarin Skeleton Preparation. SHOKRALLA N. SEDRA..... 223

A Rapid One-Step Mallory-Heidenhain Stain for Connective Tissue. JANE E. CASON 225

Notes on Technic:

Chromosome Counts in Apple and Strawberry aided by Paradichlorobenzene. HAIG DERMEN and D. H. SCOTT..... 227

Thick Sections for Embryology. RICHARD B. TIBBY and SOL BERNICK..... 227

Contains Title Page, Table of Contents and Index to Volume 25

Published by
BIOTECH PUBLICATIONS, Geneva, N. Y.

Entered as second class matter at the Post Office, Geneva, N. Y.

STAIN TECHNOLOGY

A JOURNAL FOR MICROTECHNIC

ISSUED QUARTERLY

SUBSCRIPTION PRICE $4.50 PER YEAR (U. S. CURRENCY)

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STAIN TECHNOLOGY

VOLUME 25 OCTOBER, 1950 NUMBER 4

THE MECHANISM OF THE GRAM REACTION. II. THE FUNCTION OF IODINE IN THE GRAM STAIN

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Received for publication February 27, 1950

ABSTRACT.—**Fifty-five reagents were studied as to their ability to replace iodine in the Gram stain. None gave results as good as iodine. Eight gave usable Gram preparations, and forty-seven gave negative results. Omission of the counterstain resulted in increasing to thirty-three the number of reagents giving differentiation, but this was not considered a true Gram differentiation. Many oxidizing agents were shown not to be substitutes for iodine; therefore the function of iodine must be more than to serve as an oxidizing agent. Many reagents which formed precipitates with the dye could not replace iodine; therefore factors other than precipitate formation must be involved. However, all agents which were good substitutes for iodine were both good oxidizing and dye precipitating agents. Experiments involving the study of cell membrane permeability showed that Gram-positive cells were less permeable to iodine in alcoholic solution than Gram-negative cells. This difference could not be demonstrated for iodine in aqueous solution. It was concluded that iodine served to form a dye-iodine precipitate (or complex) in the cell. Since Gram-positive cells were less permeable to iodine in alcohol than Gram-negative cells, this resulted in a slower dissolving out of this complex from Gram-positive cells during decolorization and hence a slower decolorization time. The relative solubilities of dye precipitates in alcohol and in aqueous safranin solution were also indicated as an important factor influencing decolorization. Dyes which formed highly soluble precipitates with iodine could not be used in the Gram stain. It is not proposed that the mechanism of the Gram stain is entirely one of membrane permeability; chemical factors are undoubtedly important and will be discussed in a later paper. However, it is proposed that the chemical and physical factors are closely interrelated in the Gram stain mechanism.**

Theories of the function of iodine in the Gram reaction are as

varied as the theories of the mechanism of the Gram stain itself. Many of the theories are mutually incompatible, and are based on mutually conflicting experimental results. Therefore, by necessity, any investigation into the role of iodine must begin with a repetition of previous experiments concerning agents capable of substituting for iodine in the Gram stain.

The chemical theories of the Gram stain mechanism usually assign to iodine an essential role in the formation of a Gram-positive dye-iodine-cell-substance complex. This complex is not formed in Gram-negative cells (e.g., Schumacher, 1926). Stearn and Stearn (1924 a,b, 1930) presented another concept. They believed that the essential difference between Gram-positive and Gram-negative cells was the isoelectric point of the protoplasm. Gram-positive cells were thought to have a lower isoelectric point than Gram-negative cells, and hence a greater affinity for basic dyes such as crystal violet. In this theory, iodine acted as an agent for the oxidation of certain cell substances. This oxidation lowered the isoelectric point of the protoplasm, and since Gram-positive cells possessed more of this substance than the Gram-negative, the differences in their isoelectric points were further increased. Thus, iodine served only to intensify the difference between the isoelectric points of the two types of protoplasm, and Stearn and Stearn stated that any oxidizing agent could function in the same manner as iodine.

The Gram stain has also been explained entirely on the basis of cell membrane permeability, in which case various roles have been assigned to iodine. Burke (1922) stated that while both the dye and iodine were permeable to both types of cells, the dye-iodine precipitate formed within the cells could not pass out through the membrane of Gram-positive but could pass through the membrane of Gram-negative cells. Benians (1912) stated that the difference was in permeability to alcohol. The membrane of Gram-positive cells was not permeable to alcohol, while that of Gram-negative cells was and hence the Gram-negative cells decolorized faster. He stated that iodine increased the alcohol impermeability of the Gram-positive cells. In a later paper (1920) Benians altered his view and stated that iodine formed a dye-iodine precipitate which was not readily permeable through the membrane of Gram-positive cells. Kaplan and Kaplan (1933) believed the difference to be in cell membrane permeability to iodine in alcoholic solution. According to their theory, iodine formed a dye-iodine precipitate in the cells of both Gram-positive and Gram-negative organisms. On application of the alcohol to Gram-negative cells the dye-iodine complex partially dissociated, the iodine left the cell and so did the dye. With Gram-positive cells, the iodine did not readily pass through the membrane; therefore dissociation of the dye-iodine complex, and decolorization, was much slower.

In the present work we have endeavored to determine the function of iodine by (1) a study of the Gram stain workability of all previously reported substitutes for iodine and many never before tried, (2) a study of cell membrane permeability to iodine, and (3) a study of solubilities of various dye-iodine precipitates.

Methods and Results

A. *Survey of iodine substitutes.* The Hucker-Conn (1923) modification was used for Gram staining exactly as recommended (including the use of ammonium oxalate in the crystal violet), except that other reagents were substituted for the iodine solution. Test organisms were *Saccharomyces cerevisiae, Bacillus subtilis, Neisseria catarrhalis,* and *Escherichia coli,* grown on nutrient agar slants at 37° C. for 18 hours. A large number of identical thin smears were prepared using

Table 1. Reagents Which Gave Gram Stains, Using a Counterstain

Results comparable to iodine	Results variable, but Gram stain usually obtained
*Br_2	$HgCl_2$
Br_2 (in 1% KBr)	$Hg(NO_3)_2$
Picric acid (0.1%)	
Ammonium picrate	
Sodium picrate	
Nascent iodine (KI plus H_2O_2)	
HgI_2 (in 1% KI)	
HgI_2 (in 1% $Na_2S_2O_3$)	

Notes: (*) Reagent used by Stearn and Stearn (1924)
(1) Concentrations were 1% unless otherwise indicated.
(2) All reagents in Table 1 gave heavy precipitates with crystal violet.
(3) None of the above, however, gave quite as good a Gram stain as iodine.

the same method as in the first paper of this series (Bartholomew and Mittwer, 1950). Four stains were made on different days before discarding any reagent as giving negative results. All those giving good results were tested at least 12 times. Those giving variable results were tested about 20 times. Concentrations of iodine substitutes used were 1%, unless saturated solutions were less than this amount. Occasionally a reagent in 1% concentration bleached the dye; in this case the reagent was diluted until bleaching no longer occurred.

The use or omission of the counterstain greatly influenced results. Table 1 shows that of 55 reagents tried, only 8 gave results suggesting a true Gram stain. Even these did not give as good results as the regular iodine. Table 2 shows that when the counterstain was omitted many reagents appeared to be able to replace iodine. However, a true Gram reaction cannot be obtained if the counterstain is omitted (Burke and Barnes, 1929; Bartholomew and Mittwer, 1950); therefore these results are really negative. An additional indication of the

negative nature of the results presented in Table 2 is the fact that a "pseudo Gram reaction" was possible when both iodine and counterstain were omitted (Bartholomew and Mittwer, 1950). These results do not confirm Stearn and Stearn's statement that any oxidizing agent can replace iodine, since many oxidizing agents did not work in the complete Gram stain. The failure of oxidizing agents to replace iodine was not due to their potential since failure occurred with agents both above and below iodine in potential. Attempts to

TABLE 2. REAGENTS WHICH DID NOT GIVE GRAM STAINS, USING A COUNTERSTAIN

Fair differentiation obtained only if no counterstain used. The counterstain replaces the primary dye (pseudo Gram reaction)	Slight differentiation if no counterstain used
2,4–dinitrophenol (0.5%) (±)	No reagent
*H_2O_2(–)	*$KMnO_4$ (0.1%) (+)
*$K_2Cr_2O_7$ (0.02N) (+)	$Ce(SO_4)_2$ (0.1M) (+)
NaClO (0.01%) (±)	Pyrogallol (+)
KI (+)	Hydroquinone (+)
KCl (–)	Trinitrobenzene (0.4%) (–)
$K_3Fe(CN)_6$ (0.02%) (+)	Phosphotungstic acid (0.1%) (+)
$KClO_4$ (+)	$FeCl_3$ (0.02%) (–)
$HClO_4$ (0.1%) (+)	$CuSO_4$ (–)
$SnCl_4$ (0.02%) (+)	Alum (–)
Na_2O_2 (–)	K_2PtCl_6 (0.5%) (+)
$AgNO_3$ (+)	Sulfosalicylic acid (–)
Chromic acid (+)	NH_4NO_3 (±)
Tannic acid (+)	$(NH_4)_2MoO_4$ (±)
$Na_2S_2O_3$ (±)	$Al_2(SO_4)_3$ (±)
KNO_3 (±)	Trichloracetic acid (±)
$KClO_3$ (±)	Lead acetate (±)
$NaMoO_4$ (±)	Uranium acetate (0.5%) (+)
$Hg(CN)_2 \cdot HgO$ (±)	Na_2WO_4 (±)
$KBrO_3$ (±)	KBr (±)
Uric acid (0.006%) (±)	NaCN (±)
Ferric ammonium citrate (+)	Nitrobenzene (0.2% (±)
	Dinitrobenzoic acid (±)

Notes: (*) Reagents used by Stearn and Stearn (1924) and Burke and Barnes (1929).
(1) Concentrations were 1% unless otherwise indicated.
(2) (+) = precipitate obtained with crystal violet; (±) = slight or doubtful precipitate; (–) = no precipitate.
(3) In all cases, counterstain destroyed differentiation.

reverse the action of iodine by applying reducing agents such as formaldehyde and ascorbic acid failed. Therefore, the role of iodine is not just to serve as an oxidizing agent. Table 2 also shows that experimental results consistent with those obtained by Stearn and Stearn could be obtained when the counterstain was omitted. We agree with the contention of Burke and Barnes (1929) that Stearn and Stearn erred when they used these results to explain the function of iodine in the true Gram reaction. The results confirm the statement of Burke and Barnes (1929) that the mordant must form a

precipitate with the dye which is insoluble in water but soluble in the decolorizer. Our results show however that precipitate formation itself is not sufficient since many precipitate formers did not work in the Gram stain. Work to be reported later indicates that precipitates which do not work are readily soluble in either alcohol or the counterstain and are therefore easily dissolved out of the cell. The precipitate must be neither insoluble nor very soluble in alcohol.

B. *Studies of cell membrane permeability.* The fact that crushed cells of Gram-positive organisms stain Gram-negatively has long been a strong argument in favor of the cell membrane permeability theory of the Gram stain (see Benians, 1920; Burke and Barnes, 1929). Schumacher (1926) apparently refuted this by staining Gram-positive cells with crystal violet, applying iodine, and then crushing. Alcohol applied at this stage did not decolorize the cells. Churchman (1929) in a similar experiment with yeast, reported that the extruded material did decolorize and stain Gram-negatively. We have made many attempts to reproduce the results of Schumacher, but in our experiments the stained and then crushed cells always decolorized and hence were Gram-negative. It is possible that the crushing operation resulted in a spontaneous loss of magnesium ribonucleate from the cell material. However our attempts to replate Gram-positivity to crushed cells with magnesium ribonucleate (Henry and Stacey, 1943) were always unsuccessful. Therefore the importance of the cell membrane was indicated.

TABLE 3. PERMEABILITY OF IODINE THROUGH THE MEMBRANES OF GRAM-POSITIVE AND GRAM-NEGATIVE CELLS

Procedure	Gram-positive cells: *Saccharomyces cerevisiae* *Bacillus subtilis*	Gram-negative cells: *Neisseria catarrhalis* *Escherichia coli*
1. Applied Gram's iodine. Observed at 1 minute	Stained	Stained
2. Blot dry; applied 95% alcohol; observed at 3 minutes	Remained stained	Decolorized
3. Blot dry; applied aqueous KI solution (1%). Observed at 1 minute	Decolorized	Decolorized
4. To another smear, applied iodine of same concentration as in (1) but dissolved in 95% alcohol	Not stained	Lightly stained

Our next experiments were conducted to study the permeability of iodine through the cell membranes of Gram-positive and Gram-negative cells. Permeability was followed by observing the ability of iodine solutions to stain the cells on heat-fixed slides. The reagents indicated in Table 3 were placed on the slide, followed by a cover slip, and the reaction observed with the microscope.

Table 3 is interpreted as follows: in step 1, all cells were stained because iodine in aqueous solution (I_3^-) could rapidly penetrate the membranes of both types of cells. In step 2, the iodine in alcoholic solution (I_2) was more permeable through the cell membrane of Gram-negative than of Gram-positive cells. In step 3, iodine in aqueous solution rapidly escaped from both types of cells (reverse direction from step 1). In step 4, iodine in alcoholic solution penetrated Gram-positive cells much more slowly than Gram-negative cells (reverse direction from step 2). This is a direct demonstration of the relative permeability to iodine of the cell membrane of Gram-positive and Gram-negative cells claimed by Kaplan and Kaplan (1933). This work was repeated using sixteen different species of bacteria and yeasts with identical results.

This work was also repeated using picric acid in aqueous and in 95% alcohol solutions with similar, though somewhat less differential, results.

TABLE 4. THE EFFECT OF IODINE ON THE DECOLORIZING ABILITY OF ALCOHOL

Time	Absolute alcohol plus 0.33% iodine				Absolute alcohol alone			
	Saccharomyces cerevisiae	*Bacillus subtilis*	*Neisseria catarrhalis*	*Escherichia coli*	*Saccharomyces cerevisiae*	*Bacillus subtilis*	*Neisseria catarrhalis*	*Escherichia coli*
1 min. . . .	+	+	+	+	+	+	−	−
5 min. . . .	+	+	+	−	+	+	−	−
20 min. . .	+	+	±	−	+	+	−	−
2 hrs.	+	+	−	−	+	−	−	−
26 hrs. . . .	+	+	−	−	−	−	−	−
66 hrs. . . .	±	±	−	−	−	−	−	−

Note: (+) = Gram-positive; (−) = Gram-negative.

In further experiments the concentration of the iodine in 95% alcohol was varied and the time required for staining to occur was observed. It was found that alcoholic iodine did eventually penetrate yeast cells, but rather slowly. For instance 1% iodine in 95% alcohol stained the cells in 10 minutes and 0.33% in 25 minutes. Crushed yeast stained in a few seconds as did Gram-negative cells. The permeability differences reported are therefore relative rather than absolute.

Kaplan and Kaplan (1933) reported that the addition of iodine to the alcohol used in decolorization greatly reduced its ability to decolorize Gram-positive cells. This was explained on the basis of the adverse effect the iodine would have on the equilibrium involved in the passage of iodine through the cell membrane. This experiment was repeated and the time element involved was carefully recorded. Gram stains were made on a large number of slides, and decoloriza-

tion was performed by immersing the slides in absolute alcohol containing 0.33% iodine. Slides were withdrawn at intervals and counterstained. Table 4 shows the times required to decolorize.

Table 4 is interpreted by us in a manner similar to that of Kaplan and Kaplan, but with certain modifications. In an equilibrium reaction across a membrane, iodine on the outside of the cell would retard the flow of iodine from the inside of the cell to the outside. Thus, the decolorization of both Gram-positive and Gram-negative cells would be retarded. The retardation, however, would be much greater for the Gram-positive cells since iodine in alcoholic solution is much less permeable across the Gram-positive cell membrane than the Gram-negative cells. Hence, the dissociation of the dye-iodine

TABLE 5. QUANTITATIVE ESTIMATION OF AQUEOUS AND ALCOHOLIC IODINE PENETRATION INTO GRAM-POSITIVE AND GRAM-NEGATIVE CELLS

Gram-positive cells			Gram-negative cells		
	Klett reading			Klett reading	
Organism	Aqueous iodine	Alcoholic iodine	Organism	Aqueous iodine	Alcoholic iodine
Bacillus macerans	256	73	*Aerobacter aerogenes*	60	15
Bacillus megatherium	286	111	*Pseudomonas putida*	135	92
Kurthia zenkeri	256	90	*Neisseria catarrhalis*	93	40
Saccharomyces cerevisiae	>900	17	*Serratia marcescens*	130	29
Bacillus subtilis (normal)	221	55	*Escherichia coli*	145	39
Bacillus subtilis (replated) (2)	198	62	*Bacillus subtilis* (Gram-negative) (1)	148	51

Notes: (1) *B. subtilis* was rendered Gram-negative by ribonuclease digestion, using the method of Bartholomew and Umbreit (1944). Conversion was about 90% complete.

(2) *B. subtilis* was replated to Gram-positive with magnesium ribonucleate using the method of Henry and Stacey (1943). Replating was about 75% complete.

compound within the Gram-positive cell would be retarded to a greater extent than in the Gram-negative cell.

The previous results were based on visual observation. An attempt was made to obtain more objective and quantitative information. In this experiment, the iodine extracted from the cells with KI solution in step 3 of Table 3 was measured by adding starch solution and reading the resulting blue color with the Klett colorimeter. The procedure was as follows: one loop (calibrated to contain 0.01 ml.) of cells was added to 2 ml. of 0.33% iodine in 1% aqueous KI solution, and 1 loop of cells was added to 2 ml. of 0.33% iodine in 95% alcohol. The cells were suspended by shaking for 2 minutes, and were centrifuged for 2 minutes at 6,000 r.p.m. The supernatant was poured off and the cells were resuspended in 3 ml. of 95% alcohol.

This step washes off iodine adsorbed to the surface of Gram-positive cells and extracts a major portion of the iodine from the Gram-negative cells. The cells were centrifuged again for 2 minutes and the supernatant discarded. Three milliliters of 1% aqueous KI solution were then added to extract the iodine, and the cells were suspended and shaken for 1 minute. The cells were then centrifuged for 3 minutes and the supernatant containing the extracted iodine was poured into a Klett tube. Three milliliters of 0.15% soluble potato starch solution were added, the tube shaken and read immediately in the Klett apparatus, using the 540 mμ filter. The results are presented in Table 5.

We have found that this method of quantitative iodine determination is reasonably accurate within a proper range if performed carefully and readings made immediately after adding the starch solution. The readings from 50 to 250 fall upon a straight line when graphed against micrograms of iodine. A reading of 50 represents about 1.3 micrograms of iodine per milliliter and a reading of 250 represents about 2.3 micrograms.

The interpretation of the results presented in Table 5 requires that one keep in mind that all cells were subjected to one alcohol wash following exposure to iodine. In comparing the results for Gram-positive and Gram-negative cells it is obvious that both types of cells were more permeable to aqueous than to alcoholic iodine. However, there was a quantitative difference in that the Gram-positive cells retained more of the iodine (note the column for aqueous iodine) following the alcoholic wash than the Gram-negatives. This is consistent with what would be expected from the results presented in Table 3. Unfortunately it was impossible to compare the maximum amount of iodine which entered Gram-positive with that entering Gram-negative cells, since it was impossible to eliminate adsorbed iodine on unwashed cells without influencing internal iodine. Therefore, the results presented in Table 5 are of value chiefly in that they indicate a quantitative confirmation of the visual results presented in Table 3. Additional weight is given to this data by the fact that Breinl (1920) reported that Gram-negative cells take up more iodine than Gram-positives. Table 5 has an additional point of interest since it shows that *B. subtilis* changed permeability characteristics on conversion to the Gram-negative state by ribonuclease, and reversed this change on reconversion to the Gram-positive state on replating with magnesium ribonucleate. While this experiment was strictly preliminary in nature and therefore inconclusive, it does indicate a rich field for additional research, and it suggests a way in which the chemical and membrane permeability theories as to the Gram stain mechanism might be interrelated.

C. *Solubilities of dye-iodine precipitates.* If relative permeabilities to alcohol or to iodine in alcohol were the only factors involved in

the Gram stain, any dye which is precipitated by iodine should give a Gram stain. We have shown previously, however, (Bartholomew and Mittwer, 1950) that many dyes are precipitated by iodine, yet give an inferior Gram stain or none at all. We have also shown earlier in this paper, that substitutes for iodine which give a precipitate with dye are not all suitable for use in the Gram stain. Therefore the solubility of the precipitate in alcohol or in safranin solution may also be part of the Gram mechanism. A study is now in progress of the relative solubilities of various dye-iodine precipitates. Preliminary results have shown that in general the iodine precipitates of those dyes which work well in the Gram stain, such as crystal violet and malachite green, are slightly soluble in alcohol and relatively insoluble in aqueous safranin; precipitates of dyes giving an inferior Gram stain, such as toluidine blue are moderately soluble; and precipitates of dyes which do not work, such as rhodamine B are readily soluble in both alcohol and aqueous safranin. Dyes which do not give a precipitate are useless. Precipitates made with picric acid (which gave a good Gram stain, but not as good as iodine; see Table 1) showed an analogous trend, though in general these precipitates were more soluble than iodine precipitates. Potassium iodide (see Table 2) precipitated these dyes but did not give a Gram stain. It was found that dye-KI precipitates were all very soluble. Quantitative data and fuller discussion of this work will be given in the next paper of this series.

Discussion and Summary

The results presented in this paper clarify the question as to the role of iodine in the Gram procedure. Some of the confusion resulting from previous conflicting experiments was resolved. The statement of Stearn and Stearn (1924) that any oxidizing agent can serve the same function as iodine has been shown to be wrong. Stearn and Stearn's results could be duplicated, but since they omitted the counterstain they were not studying the true Gram reaction (Burke and Barnes, 1929; Bartholomew and Mittwer, 1950). Stearn and Stearn's concept of iodine functioning to lower the isoelectric point of cell protoplasm is not acceptable for reasons other than the failure of other oxidizing agents to function. The concept of isoelectric point influencing dye affinity is sound and can easily be confirmed. However, the application of this concept to the Gram mechanism does not seem to be valid for a variety of reasons. The present authors have Gram-stained a mucoprotein preparation with an isoelectric point of 1.8 only to find it Gram-negative. Bacterial cells which have been methylated (Bartholomew, Evans, and Nielson, 1949) and thus have their ordinary isoelectric points destroyed, will still give Gram differentiation if certain acid dyes are used in the Gram procedure.

Our results agreed best with those of Burke and Barnes (1929). All usable iodine substitutes formed a good precipitate with the dye. However, ability to form a dye precipitate was not the only necessary quality of the iodine substitute since many precipitate formers did not function. It will be shown in a later paper that the solubility of the precipitate in alcohol and in the counterstain was an important factor. The disagreement of Burke and Barnes with Stearn and Stearn as to the ability of hydrogen peroxide to replace iodine was due solely to the failure of the latter workers to apply a counterstain.

Our work agreed with Kaplan and Kaplan (1933) as to the relative impermeability of Gram-positive cells to iodine in alcoholic solution. This appeared to be another important characteristic of iodine, which gave this substance its peculiar Gram differentiation ability in that it slowed the dissolution of the dye-iodine precipitate from Gram-positive cells. Substitutes for iodine, such as picric acid, were shown to possess both the dye precipitating ability and differential permeability in alcoholic and aqueous solutions.

Burke and Barnes (1929) and Benians (1920) have claimed that there is no such thing as a Gram-positive dye-iodine-cell-substance complex. The present work does not disprove the existence of such a complex, nor does it confirm it. We feel that such a complex probably exists, particularly for Gram-positive inclusion bodies in the cells. Certainly the role of magnesium ribonucleate in the Gram-staining properties of the cell is well known (Henry and Stacey, 1943; Bartholomew and Umbreit, 1944). However, we feel that cell membrane permeability data cannot be ignored when the Gram stain mechanism is considered. The invariable Gram-negative nature of crushed cells, and the inability to replate magnesium ribonucleate on to this crushed material, strongly indicate an important role for the intact cell membrane. It is possible that magnesium ribonucleate is important in that it influences cell membrane permeability, probably by functioning as an essential part of the molecular architecture of the membrane of Gram-positive cells. We believe that the reports of cell independent Gram-positive material are subject to doubt.

The problem of differential permeability of Gram-positives and Gram-negatives to alcohol has not been considered in this paper. No rigorous method of studying this problem was available. However, previous work (Bartholomew and Mittwer, 1950) indicated that both Gram-positive and Gram-negative cells were decolorized with alcohol when iodine was omitted from the Gram stain. A difference in the decolorization of the two types of cells was noted, but it was not sufficient to account for Gram differentiation. Therefore, this factor would probably be contributory in only a minor degree to the Gram stain mechanism.

The concept of differential permeability of iodine in alcoholic, but not aqueous, solution for Gram-positive cells is consistent with the

well known influence of the addition of water to the decolorizing alcohol on its decolorization of Gram-positive cells (Neide, 1904; Burke, 1922).

ACKNOWLEDGMENT

The work presented in this paper was made possible by generous research grants from the Biological Stain Commission and the Office of Naval Research. The authors wish to express their appreciation of the assistance and advice offered by Dr. H. J. Conn.

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DYE EXCHANGE IN BACTERIAL CELLS, AND THE THEORY OF STAINING

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Received for publication Mar. 23, 1950

Abstract.—**Bacterial cells were stained in sequence, and at various pH's, by 22 different basic dyes. It was found that any dye could replace another already present in the bacterial cell. This replacement was shown to act according to mass action laws for reversible reactions, and hence was influenced by concentration of reagent and time of application. Since basic dyes are also known to react at carboxyl group sites, the phenomena of staining of bacterial cells by ordinary basic dyes must be a chemical adsorption exchange reaction.**

In an earlier paper (Bartholomew, Evans, and Neilson, 1949) direct proof was given of the chemical nature of the staining reaction for bacterial protein. The principal reaction for basic dyes was shown to occur at the sites of carboxyl groups. This paper presents additional studies as to the nature of the dye-carboxyl group bond, as determined by methods involving the replacement of dye on the carboxyl group by other dyes, and by inorganic salts.

The ability of dyes and other reagents to replace the dye on a stained bacterial cell has been known since the days of Weigert (in Deletang, 1933). However, this phenomenon has been little studied. Lasseur and Schmitt (1927) observed that the counter stain would replace the primary stain in the gram reaction if given sufficient time. Coudroy (1929) reported that neutral salts could replace alcohol as the decolorizer in the gram reaction. Kaplan and Kaplan (1933) found that if *Escherichia coli, Sarcina* and several spore-forming soil bacteria were stained first with crystal violet, and then with safranin, the safranin replaced the crystal violet first in the *Sarcina* and soil spore-formers, and lastly in *E. coli*. Up to the present time, a sound explanation of these replacement reactions has been lacking. This paper presents such an explanation. The general mechanism of staining of bacterial cells by basic dyes is also explained.

Methods

Escherichia coli, Micrococcus aureus, Pseudomonas pavonacea, and *Bacillus subtilis* were used as test organisms. Distilled water suspensions of 24-hour nutrient agar slant cultures were used to prepare the heat-fixed slides.

Distilled water solutions of the dyes were prepared in the concentrations stated in the experiments. In one experiment the dyes were prepared in phosphate buffers at pH 5, 7, and 9. Various dye sequences were obtained by staining with the first dye, blotting dry, and

then staining with a second dye. This process was repeated for each dye in the sequence. Staining time was always one minute, unless otherwise indicated. In experiments involving dye replacement by salts, and dilute dyes, the primary stain was applied to the slides which were then blotted and placed in a Coplin jar containing the

TABLE 1. REPLACEMENT OBTAINED WITH VARIOUS STAIN SEQUENCES*

STAIN SEQUENCE	ORGANISM			
	E. coli	*M. aureus*	*P. pavonacea*	*B. subtilis*
MB	(100)	(100)	(100)	(100)
MB–CV	40	100	100	100
MB–CV–S	90	90	100	100
MB–CV–S–MG	60	100	100	100
MB–CV–S–MG–MB	60	100	100	100
MB–CV–MG	90	100	100	100
MB–CV–MG–S	90	100	100	100
MB–CV–MG–S–MB	95	100	100	100
MB–S	80	85	100	100
MB–S–MG	50	100	100	100
MB–S–MG–CV	—	100	100	100
MB–S–MG–CV–MB	80	100	100	100
MB–S–CV	100	100	100	100
MB–S–CV–MG	90	100	100	100
MB–S–CV–MG–MB	60	100	100	100
MB–MG	90	100	100	100
MB–MG–S	90	100	100	100
MB–MG–S–CV	—	100	100	100
MB–MG–S–CV–MB	90	100	100	100
MB–MG–CV	100	100	100	100
MB–MG–CV–S	60	99	100	100
MB–MG–CV–S–MB	90	100	100	100
S	(100)	(100)	(100)	(100)
S–CV	100	100	100	100
S–MB	90	100	100	100
S–MG	70	100	100	100
CV	(100)	(100)	(100)	(100)
CV–S	100	100	100	100
CV–MB	99	100	100	100
CV–MG	100	100	100	100
MG	(100)	(100)	(100)	(100)
MG–S	100	100	100	100
MG–CV	100	100	100	100
MG–MB	100	100	100	100

*Figures represent the percentage of cells bearing the color of the final stain in a sequence.

MB—methylene blue 1% aqueous solution—National Aniline Cert. No NA-27
CV—crystal violet 1% aqueous solution—National Aniline Cert. No. NC-32
MG—malachite green 1% aqueous solution—National Aniline Cert. No. NMg-13
S—safranin 1% aqueous solution—National Aniline Cert. No. NS-18

replacement reagent. Slides were then removed at the indicated time intervals, and observed.

RESULTS

The first series of experiments was conducted to determine the extent of the ability of one basic dye to replace another in the bacterial cell. One percent aqueous solutions of crystal violet, methylene blue, malachite green, and safranin were used in 34 different dye se-

quences. Table 1 shows that in all stain sequences the cells took the color of the final dye applied. The results for *E. coli* were erratic in that the dye exchange was incomplete in many cases. However, when staining time was increased to five minutes, complete replacement by the final dye was obtained. The stain sequences used were sufficiently varied to show that replacement was not due to an increasing affinity for carboxyl groups by each succeeding dye.

The effect of the pH of the dye solution on replacement was studied by preparing 0.5% solutions of crystal violet, methylene blue and safranin in phosphate buffers of pH 5, 7, and 9, and applying them in over 30 possible sequences. The results obtained in this experiment were similar to those of the previous experiment. One dye replaced another regardless of sequence or pH. *Escherichia coli* again

TABLE 2. OTHER DYES USED IN REPLACEMENT EXPERIMENTS

NAME OF DYE	DYE GROUP	AQ. SOL.	DYE DATA
New fuchsin	Triphenyl	.5%	Nat. Aniline—12096
Hoffman's violet	Triphenyl	.5%	Nat. Aniline—10799
Methyl green	Triphenyl	.5%	Cert. No. NG-20
Para-rosanilin-chloride	Triphenyl	.5%	Cert. No. PR-21
Rosanilin ZnCl double salt	Triphenyl	.4%	Cert. No. PR-10
Victoria blue B	Phenyl methane	.4%	Nat. Aniline—10098
Methyl violet B	Triphenyl	.4%	Unknown
Nile blue sulfate	Quinone-imine	.5%	Cert. No. NNb-3
Cresyl violet	Quinone-imine	.5%	Cert. No. NW-17
Azure II	Quinone-imine	.5%	Nat. Aniline—5982
Toluylene blue	Quinone-imine	.5%	Nat. Aniline—11659
Thionin	Quinone-imine	.5%	Cert. No. NT-14
Acriflavine (neutral)	Xanthene	.5%	Nat. Aniline—12711
Rhodamine B	Xanthene	.5%	Nat. Aniline—11534
Janus green B	Mono-azo	.5%	Cert. No. NJ-2
Bismarck brown	Diazo	.5%	Cert. No. NN-11
Chrysoidin Y	Mono-azo	.4%	Unknown

exhibited variability, but it was again possible to remedy this by increasing the staining times to five minutes.

Differences in ability to replace were noted. When a slide was stained initially with a basic dye at pH 9, and then subjected to a second basic dye at pH 5, replacement was considerably slower than when the conditions were reversed. This result would be expected because of the well known influence of pH on the affinity of basic dyes for bacterial protoplasm. (Stearn and Stearn, 1924; McCalla, 1941b).

The experiments were next enlarged to include 17 additional basic dyes. These are listed in Table 2. These were applied as before, and in at least thirty different sequence combinations. In all instances dye replacement occurred. Therefore, the ability for one basic dye to replace another in the bacterial cell is a general phenomenon since no exceptions were found.

The next experiment was conducted to determine the influence of dye concentrations, and time of application of the second dye on re-

placement. Table 3 presents results typical of those obtained. It was found that the more dilute the second dye, the longer the time required for replacement. The results suggest the applicability of the mass action law for a reversible reaction.

The ability of inorganic salts to replace dyes was studied next. Table 4 shows that salts will replace a dye if given sufficient time. However, the replacement by salts was very slow as compared to the replacement of one dye by another, and the results varied with the salt used. This would indicate that ionic affinities influence the replacement time. Increasing the concentration of the salt speeded the replacement process. Again, the ability of a salt to replace a dye suggested the applicability of the mass action laws.

The role of osmotic pressure, if any, in dye replacement was studied by exposure of slides stained for one minute by 0.01 *M*

TABLE 3. REPLACEMENT OF 0.5% METHYLENE BLUE BY DECREASING CONCENTRATIONS OF SAFRANIN

M. aureus used as test organism

PER CENT SAFRANIN	TIME							
	Minutes				Hours			
	1	5	10	25	1	5	10	20
0.5.....	**4 + R**							
0.05.....	**4 + R**							
0.005.....	1 + V	2 + V	3 + V	4 + V	**4 + R**			
0.0005.....	4 + B	3 + B	1 + V	1 + V	2 + V	3 + V	4 + V	**2 + R**
Water control.....	4 + B	4 + B	4 + B	4 + B	4 + B	3 + B	3 + B	3 + B

Staining intensity is designated as 1 + to 4 +.
Complete replacement is indicated by bold faced type.
R—red V—violet B—blue

methylene blue, safranin and crystal violet, to 1%, 10% and 20% glucose for one minute. It was found that the glucose failed to replace the dye in the cells despite the high osmotic pressures used. Therefore, dye replacement in these experiments was due to other than osmotic factors.

Mordants, such as iodine, create conditions very different from those presented in this paper. Iodine vastly increased the replacement times. For instance, when 1% crystal violet was used to stain *B. subtilis* it was replaced by 0.005% safranin in less than five minutes. However, when *B. subtilis* was stained for one minute with 1% crystal violet, followed by a one minute exposure to Gram's iodine, it took two hours to replace this complex with 0.25% safranin. The problem of staining when mordants are used is much more complicated than the simple staining reactions presented here. Mordants complicate the picture by forming mordant-dye precipitates, and thus cell membrane permeability problems become involved; and by influencing the staining ability of a dye compound. The problem

of mordants is outside the scope of this paper, and will be presented elsewhere.

Discussion

The experiments just presented result in a clearer concept of the staining mechanism as occurring in bacterial cells. Earlier work (Bartholomew, Evans and Neilson, 1949) has shown that basic dyes unite primarily with free carboxyl groups in the bacterial cell substance. Thus, the staining reaction occurs at a specific chemical site. The experiments present in this paper, show that the carboxyl group-dye bond is a reversible one, and that the dye can be removed from the

Table 4. Replacement of Crystal Violet (0.01 *M*) by 0.1*N* and 0.01*N* Salts. Effect of Time and Concentration of Salt.
M. aureus used as test organism

Salt	Time											
	Minutes				Hours							
	1	5	10		1		2		3	4	5	
Conc (*N*)	0.01	0.01	0.1	0.01	0.1	0.01	0.1	0.01	0.01	0.01	0.1	0.01
$NaCl$	4	4	4	4	3	3	3	3	2	1	0	1
$CaCl_2$	3	3	2	2	1	1	0	0	0	0	0	0
$CuCl_2$	3	3	2	2	0	1	0	0	0	0	0	0
$FeCl_2$	4	3	2	2	2	1	0	1	1	0	0	0
$MgCl_2$	4	4	2	3	2	2	0	2	1	1	0	1
$BaCl_2$	4	4	3	4	2	3	0	1	1	1	0	0
$StCl_2$	4	4	–	3	–	2	–	1	1	0	–	0
HOH	4	4	4		4		4		4	4	3	

Numbers refer to intensities of color due to crystal violet:
0 = no color
4 = color of stained slide not exposed to salt.
The primary dye was applied for one minute.

carboxyl group by other dyes, and by ionic salts, if conditions of concentration of reagent, time of action, and chemical affinity are proper. In studies of the dye uptake of bacterial cells to be published later (Bartholomew and Finkelstein[1]) it was found that even though the cells were in great excess, only about 90% of the dye was taken up. This would be expected of a reversible reaction such as discussed in this paper. This fact, together with the influence of concentration of reagent, and time of application, definitely suggest the functioning of mass action laws in the staining of bacterial cells. Staining, then, has been shown to be due to a chemical adsorption exchange phenomenon, as postulated earlier by McCalla (1941a), and Stearn and Stearn (1930).

The experiments on the ability of salts to replace dyes were not conducted in a way to produce very accurate quantitative results. However, the differences observed in the ability of various salts to

[1]Manuscript in preparation.

replace dyes are consistent with the observations of McCalla (1940) as to the ability of various salts to replace magnesium adsorbed on bacterial cells. McCalla found that sodium and potassium chlorides were poor replacers of magnesium, while calcium and barium chlorides were good. On the basis of the results presented in this paper, the same can be said regarding the ability of these salts to replace dye in the cell. McCalla postulated that magnesium was adsorbed at carboxyl group sites; if so, then magnesium ion replacement and dye replacement by salts would be identical in principle.

The ability of dyes to replace each other has several interesting applications. The Shaeffer-Fulton stain can be explained on the basis of the safranin replacing the malachite green faster in vegetative areas of the cell than in the spore. The validity of this interpretation was demonstrated in an experiment in which we allowed the safranin to remain on the slide for twenty minutes. In this case the safranin replaced all of the malachite green including that in the spore. Bartholomew and Mittwer (1950) have clearly demonstrated that dye replacement is an important action of the safranin when it is used as a counter stain in the gram stain.

The data herein presented apply only to the staining of bacterial cells with ordinary basic dyes, and undoubtedly other mechanisms of staining exist in some cases. The solubility theory of Holmes (1926), and Conn and Holmes (1928) undoubtedly is applicable to special instances such as the staining of fat with sudan dyes.

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A SIMPLE HISTOCHEMICAL REACTION FOR ALDEHYDES[1]

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Received for publication March 20, 1950

ABSTRACT.—**A study has been made on the possibility of replacing leucofuchsin by colored basic fuchsin for the histochemical demonstration of aldehydes. Several tissues from mammals and various pertinent fixatives were used. Aldehydes were freed from carbohydrates by oxidation and from thymonucleic acid by hydrolysis.**

It was found that the colored form and not necessarily the leucoform of basic fuchsin can be used histochemically in demonstrating aldehydes. The technic used is as follows: (1) Treat with 1.0 – 0.5% H_5IO_6 (or in 1% KIO_4 in *M*/1 H_2SO_4) for 5 to 10 min. and wash thoroughly. For thymonucleic acid hydrolize with *N* HCl 5 min. at room temperature, 10 min. at 60°C. and 5 min. at room temperature. (2) Stain for 2 – 3 min. with 0.05% basic fuchsin in 5% ethanol, 3% phenol. (3). Transfer immediately to 1 or 2 changes of 1% sodium bisulphite or potassium metabisulphite in 0.1 – 0.2 *N* H_2SO_4 for a total of 5 min. (4) Rinse with water and treat with *M* H_2SO_4 in 95% ethanol for 3 – 5 min. 6. Wash thoroughly in water and dehydrate, clear, and mount. For glycogen and mucin the following counterstaining solution is recommended: orange G, 0.25 g.; light green SFY, 0.10 g.; phosphotungstic acid 0.50 g.; 50% ethanol, 100 ml.; glacial acetic acid, 0.25 ml.

The purpose of this paper is to present briefly evidence on the identity of results obtained by the use of colored fuchsin, according to a technic previously reported (Arzac, 1948), and those obtained with its leuco-form, the Schiff's reagent, for the demonstration of tissue aldehydes.

Inspired by reports by Lillie (1947), Hotchkiss (1948), and McManus (1948), this technic was evolved to simplify the procedure, lessen the cost, and shorten the time consumed as compared to any other known stable method and especially when Schiff's reagent is used for the histochemical demonstration of carbohydrates, mainly of glycogen. An extensive study has revealed that it can be proposed as a general histochemical reaction for aldehydes. Although its chemistry is perhaps somewhat different from that involved in the Schiff's reaction, both are probably similar in their essential mechanism. Attention is called to the considerable economy of time, money and

[1]At the first Meeting of The Histochemical Society on March 24, 1950, in Philadelphia, a brief of this paper was prepared and presented by Dr. R. D. Lillie, to whom the author is very much indebted for his many courtesies.

effort that it eventually brings; in our belief, it represents a substantial practical advantage over the classical method. A description of the technic will be found in subsequent paragraphs of this paper.

Methods

The tissues and fixatives used were many, as listed below. Schiff's reagent prepared according to Lillie (1947) and some times according to Rafalko (1946) was systematically used in parallel slides as the fundamental checkup of experimental procedures. Since special emphasis was placed on tissue carbohydrates, digestion with saliva was also performed to differentiate glycogen from other substances of the same chemical group. For the study of thymonucleic acid, hydrolysis in normal hydrochloric acid was standardized as follows: 5 min. in cold HCl; 15 min. at 60° C. and 5 min. in cold HCl. No rinsing in water followed.

Organs and tissues studied. These came from human and rodents and were the following: liver, endometrium, ovary, kidney, cornea, intestine, stomach, cervical mucosa, decidua, placenta, mammary glands, testicle, vaginal mucosa, and vaginal smear, thyroid, and the following tumors: chondroma, cervical adenocarcinoma, mammary adenoma, uterine fibroma, and fibrosarcoma. About 1000 specimens have been studied so far. After fixation and dehydration the tissues were embedded in paraffin. Besides this, moistened starch smeared on slides and covered with collodion, was also utilized.

Fixatives used. Alcohol, alcohol-formol-acetic, with and without picric acid and with or without mercuric chloride, unbuffered formol, 5% aqueous $CoNO_3$ and Zenker.

A. Experimentation on carbohydrates

An enumeration follows of the items experimentally studied in relation to this subject:

1. Procedure of oxidation:
   (a) 1% aqueous solution of periodic acid (H_5IO_6) 5 min. to 24 hr.
   (b) sodium periodate and nitric acid, according to Lillie (1947), 10 min.
   (c) 1% potassium periodate (KIO_4) in $M/1$ sulfuric acid
   (d) saturated solution potassium permanganate with and without HNO_3 followed by bleaching with oxalic or sulfurous acid
   (e) 10% chromic anhydride, 10 to 45 min.
2. Role on the fuchsin of:
   (a) mordants (aniline water and phenol were studied)
   (b) solvents (water, alcohol)
   (c) pH (oxalic, hydrochloric and sulfuric acid at various concentrations)

3. Role of $NaHSO_3$ or $K_2S_2O_5$ (with or without added acid), either *in* the staining solution, or *after* the staining solution.
4. Acid fastness of the substances and structures demonstrated

B. EXPERIMENTATION ON THYMONUCLEIC ACID

1. Role of fixatives
2. Role of hydrolysis

C. EFFECT OF BLOCKING THE PROBABLY ACTIVE CHEMICAL GROUPS

Following Delamater (1948) the experiments below were devised:

1. In a test tube experiment, about 2 millionths of a gram of formaldehyde, added to a bath of strong sulfurous acid, blocked the decolorization of 0.00005 g. of fuchsin which became deep violet as time passed, while a 10% solution of the same sulfurous acid bath readily decolorized a fuchsin solution with a concentration more than 10 times greater in the absence of formaldehyde in less than 5 minutes.
2. Duplication of the experiments above stated (see A and B) with formol-fuchsin and with crystal violet gave consistently negative results.

Results and Comment

The data that originated from the experimentation with the preceding items have led to the following technic, which confirms with slight modifications the one previously reported:

1. Periodic acid solution (0.5 to 1%) 10 or 5 minutes, (carbohydrates are not burned in 24 hours in a 10% soluton of this acid. Any of the oxidative procedures mentioned above can be used according to the results one desires to obtain);
2. Wash thoroughly in running water and stain 2–3 minutes with the following:

basic fuchsin	0.05 g.
96% ethanol	5 ml.
aniline water or 3% aqueous phenol	to 100 ml.

3. Immerse *directly* for a total of 5 to 10 min. in one or more (2 and often even one is enough) changes of:

10% sodium bisulfite or potassium metabisulfite	1 volume
M/1 sulfuric acid	half to 1 volume
distilled water	to 10 volumes

4. Rinse in water and treat 3–5 min. in M/1 sulfuric acid in 96% alcohol
5. Wash thoroughly and counterstain as desired.

The following formula gives good general contrast in green with blood and (if not stained with hematoxylin) nuclei in yellow particularly after fixatives containing picric acid. Collagen stains green and reticulum may be changed to green or reinforced to a deep bluish violet. The hue of the nuclei may change slightly in "Feulgen's type" reactions.

Counterstaining solution:

Orange G	0.25 g.
Light green	0.10 g.
Phosphotungstic acid	0.50 g.
50% ethanol	100 ml.
Glacial acetic acid	0.25 ml.

(This solution can be diluted at will)

Stain 1 to 3 min. according to the intensity of contrast desired. Rinse in water and dehydrate in 96% alcohol etc.

The times given herewith need not be rigidly followed. They can be considerably shortened or lengthened without serious danger of a bad result, but those given seem to be very convenient; 96% alcohol in step 5 of the technic is not absolutely essential. Lower grades and less acidity can safely be used from a practical standpoint and in many cases totally omitted. However, it is recommended on theoretical grounds.

In all cases, the results obtained with the colored form of the fuchsin as used in the preceding technic were identical to those obtained with the leucoform. The only difference (and this cannot be stated with absolute certainty) is a somewhat more reddish hue of the substances demonstrated with the former which can be modified almost at will, and turned to a deep blue before mounting, by adjustment of the final pH of the tissue when either or both types of staining procedure are finished.

SUBSTANCES AND STRUCTURES IDENTIFIED

Glycogen in: sertoli cells of human testicle; liver (mouse and rat); endometrium (human and mouse); cervical mucosa (human); cornea (rabbit); vagina (human); placenta (human, rabbit); corpus luteum (rabbit); muscular tissue from several sources; chondroma (human).

Mucin in: human vaginal, cervical, and endometrial mucosae.

Glycoprotein in: follicular cells and fluid (rabbit); colloidal substance of thyroid (rabbit); mammary alveoli (human adenoma).

"Hyaline" and other structures: reticulum, cilia, and arterial elastine (in fixative containing mercuric chloride) fibrin, basal membranes, mitosis (human cervical adenocarcinoma)

The accompanying figures illustrate and exemplify some of the results obtainable. Mordants seem to be important although not absolutely indispensable for the colored form to work. Aniline water

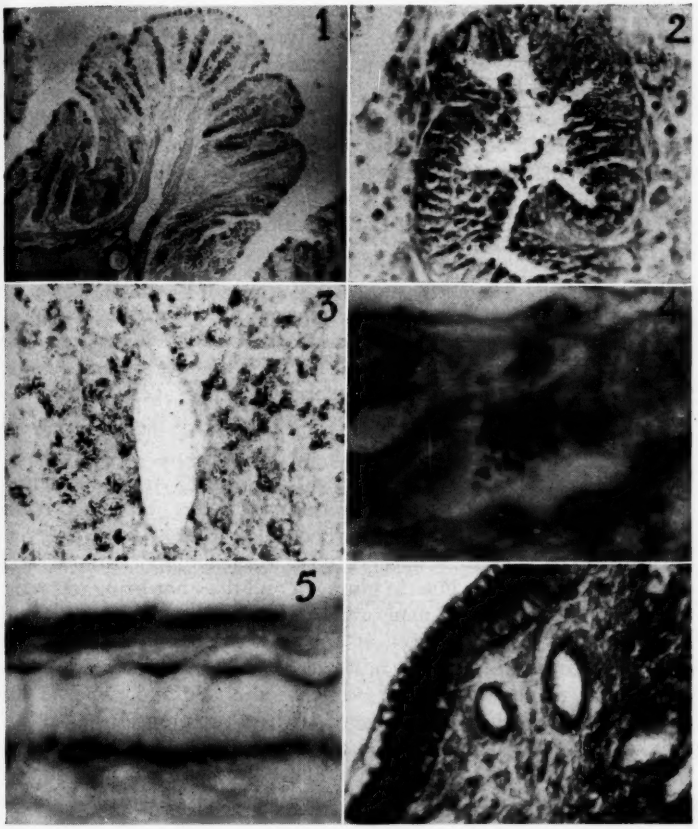

FIG. 1. — Rabbit's intestine showing goblet cells and pale reticulum. No counterstain used.

FIG. 2. — Glycogen in a human endometrial gland. Photographed through a green filter.

FIG. 3. — Central zone of mouse liver. Glycogen, but no reticulum, is clearly seen. (Green filter)

FIG. 4. — Granules of polysaccharide digestible with saliva in a corpus luteum cell from rabbit. Counterstained with hematoxylin. Magnification 920×.

FIG. 5. — Cornea of rabbit showing positive substances digestible with saliva. Amplified from a microphotograph taken at 1000×.

FIG. 6. — Mucine, basal membrane, and perivascular reticulum. Polyp of cervix fixed in formol.

has certain advantages but does not preserve the reagent as phenol does. An excess of alcohol in the solvent tends to bring out cell membranes. Too acid a medium (addition of excessive mineral acid to the dye solution) hinders the reaction with either form of fuchsin and is not necessary in the preparation of Schiff's reagent if fuchsin and sulfite are in adequate proportions. The sulfurous acid bath is more conveniently prepared with potassium metabisulfite and sulfuric acid.

The chemical similarity of both reactions is evidenced by the following:

1. (a) *Specific* oxidation by periodic acid [(Hotchkiss, 1948), or nonspecific by chromic anhydride or potassium permanganate] or specific HCl hydrolysis, both of which liberate aldehydes from the appropriate substrate, are essential for both reactions to take place, as well as the presence of H_2SO_3 *in* the staining reagent (Schiff's) or *immediately after* its use. When one or the other or both are omitted no reaction is obtained.

2. The hue of the color elicited, as well as the type of substances or structures revealed by both procedures, varies according to the type of oxidant or fixative used as reported by Lillie (1947) for leucofuchsin but always in a parallel way. For example, chromic acid oxidation gives redder hues in glycogen and does not bring out reticulum nor ovum or basal membranes either in our technic nor with leucofuchsin. While glycogen is not as well demonstrated by any of the existing technics after aqueous or alcoholic fixatives, considerably larger amounts appear after periodic acid and either of the two forms of fuschin as compared with other procedures, and in this case the amounts are the same for both types of reactions. No mordanting effect of formaldehyde is necessary for the demonstration of thymonucleic acid. In fact $CoNO_3$ gives an excellent fixation for both reactions.

The above *in vitro* experiments with smears of starch and the blocking of NH_2 groups of fuchsin and the uselessness of dyes that have them blocked, further stress Delamater's (1948) viewpoint on the chemistry of Schiff's reaction and our assumption about the similarity of this one and the one above described. In both cases positive reactions resist the action of diluted NaOH, a test for "true" Feulgen reactions (Glick, 1949).

There seems to be no danger of demonstrating acid-fast substances with the colored form, because the concentration of the dye and the time and temperature in which it acts are considerably below those required and the treatment with alcoholic $M/1$ H_2SO_4 in the conditions of our procedure rule out the theoretical possibility. In fact substances positive with our technic stand more than 24 hours in the sulfurous acid and more than 8 days in $M/1$ concentration of alco-

holic H_2SO_4 without total decolorization, which is also true for the Schiff's reaction, but not for any acid fast substance known by the present author. Sputum known to be positive for tuberculosis with the classical Ziehl-Neelsen procedure is always negative with our method. The present author does not feel compelled to discuss any aspect on specificity, since the results obtained are such as to make this a discussion of the widely disputed specificity of Schiff's reaction itself.

Summary and Conclusions

1. A simple histochemical reaction involving the direct use of colored basic fuchsin instead of leucofuchsin has been experimentally studied.

2. The results obtained were identical to those of the classical method in all experimental conditions set up to test essential and non-essential steps for both procedures.

3. It is concluded that the technic can be proposed as a general histochemical reaction for aldehydes in mammal tissues.

4. Its main advantages are: less expensive, simpler and shorter.

Acknowledgments

Acknowledgment is made of the technical assistance rendered by Miss Liliam G. Flores, Q.F.B., and Mrs. E. Velez De Mateos, M.D.

ADDENDUM

Further study of the technical steps of the colored fuchsin technic has revealed that: (a) carbolfuchsin instead of aniline fuchsin is to be preferred since aniline fuchsin does not demonstrate glycoprotein as well; (b) the sulfuric-acid-alcohol treatment can be omitted if the concentration of the sulfurous acid bath in step 3 is increased. If a nearly saturated solution of potassium metabisulfite is used as a stock solution for preparing the sulfurous acid bath, the same proportions and same time (approximately 5 min.), as herewith given in step 3 of the technic, will elicit identical results making unnecessary the sulfuric-acid-alcohol extraction. Any traces of excess fuchsin that could have remained are eliminated by the alcohols used in dehydration. The choice of one way or the other may be left as a matter of personal preference.

Periodic acid and sodium periodate were from Frederick Chemicals and potassium periodate from Baker.

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THE NEW DOMESTIC CRESYL ECHT VIOLET

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Received for publication April 29, 1950

One casualty of the late war was the excellent Grübler's cresyl echt violet. Where permanence was required the slightly inferior quality of the stain (as compared to the rapidly fading buffered thionin) was not a drawback. For the past two years or so, Coleman

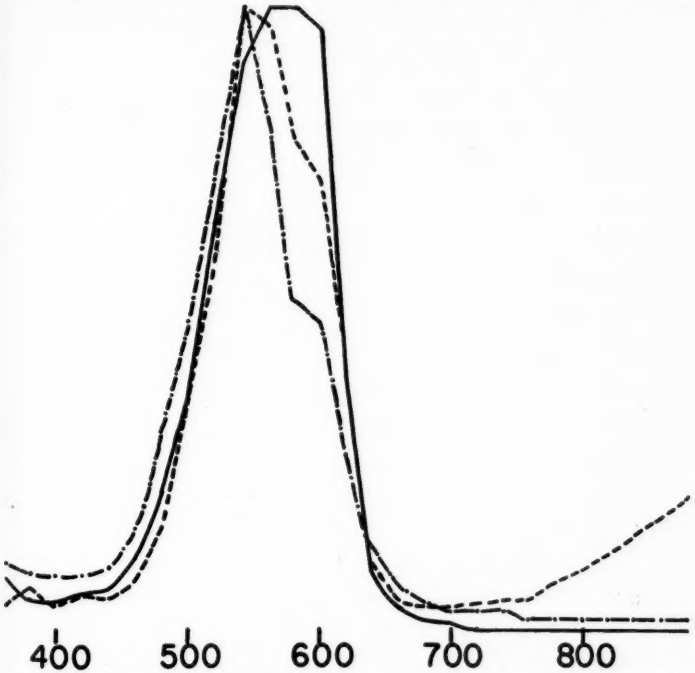

FIG. 1.—Absorption spectra of three cresyl echt violets. Domestic —; Grübler (originally sold in U.S.A.) —.—; Grübler (originally sold in Italy) — — —

and Bell have been attempting to produce a substitute for the German product. To date we have tested four of their preparations. While all gave acceptable stains, the final batch tested appears to be quite satisfactory. We have compared this product with four samples of the pre-war dye. The aqueous solution of the domestic dye was bluer than the others, and on high dilution (1:10,000 approximately) did not become a pinkish-lavender like the others. Absorption

spectra indicated that probably some of the pre-war samples were identical. Fig. 1 shows absorption spectra for the domestic product and for two of the pre-war Grübler products. The domestic product is shown with a solid line, the dash line is that of one of two similar products obtained from Italy, and the dot-dash line is that of one of two samples originally sold in this country. The curves are similar but have slightly different maxima. The Grübler products obtained from Italy have a slight absorption at higher wave lengths in addition to the maxima at 540.

The quality of the stains was determined on formalin-fixed celloidin-embedded monkey cortex and on formalin-fixed paraffin-embedded guinea pig spinal cord. All staining was similar using a regressive technic with acid-differentiation when necessary. Nissl granules stained blue-violet with the domestic product as compared to the reddish-violet of the Grübler dyes. The background in all had approximately the same very pale blue color. Celloidin retained a pinkish tint with the German products, but only a much fainter blue with the domestic.

Susceptibility to fading was tested by exposing the slides of guinea pig spinal cord to ultraviolet light. They were placed 7 inches from the tip of a 275 watt General Electric R S Sunlamp for 12 hours. This exposure produced no discernible change in the staining, but a similarly treated slide stained with buffered thionin was so faded as to be valueless.

In order to check metachromasy, a series of trachea sections were stained with the five dyes. In all cases there was good differentiation between mucin and the formed elements. With each of the dyes the range in color was of approximately the same magnitude with minor shifts of the entire band in sections stained by the various Grübler cresyl echt violets but with a definite shift towards the blue with the domestic product.

Since the slight difference in color is of no importance, it is suggested that the new domestic dye will prove an acceptable substitute for the German product.[1]

[1]*Note by Editor:* A sample of this domestic cresyl violet, received from the authors of this paper, was tested by another neurohistologist, who does not agree with the above conclusions as to its being a good substitute for the German product. It should be remarked, however, that the old German product is not believed to have been entirely constant; and these two sets of investigators may be comparing the new domestic cresyl violet with German samples that were not alike.

A CHROME-ALUM PREPARATION FOR DELICATE AND DIFFICULT FIXATIONS

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Received for publication April 13, 1950

ABSTRACT.—**A chrome-alum fixative is recommended as a reagent for general use. The basic formula is: C.P. chrome-alum, 3 g.; 40% formaldehyde, 30 ml.; glacial acetic acid, 2 ml.; distilled water, 238 ml. This fixative permits easy sectioning of yolk-rich amphibian embryos. It can be used to make permanent slides of *Euglena* showing the flagellum. It is a satisfactory fixative for insect larvae and fixes sharply the slime droplets of Planarians. Fixation should not exceed two hours or the material being fixed will swell. Rinses of 70% alcohol or water may follow the fluid. The fluid keeps well, does not harden tissues and gives good cytological detail.**

A fixative employing chrome-alum as a component has been found to give exceptional results with several types of animal structures. The basic formula of the fixative is: chrome-alum, 3 g.; 40% formaldehyde, 30 ml.; glacial acetic acid, 2 ml., and distilled water, 238 ml. This formula was arrived at empirically with the intent of keeping the reagents at the minimum amounts necessary for the desired action. When the fixative was found to perform satisfactorily, manipulation of the quantities was carried no farther.

This fixative may be followed by rinses of water or 70% alcohol without apparent difference in effect. After either rinse there is no evidence of deposits of foreign material in fixed tissues. It is suggested that the size of objects to be fixed with this fluid be limited to the quantity that may be totally reacted upon in from 1 to 2 hours. Immersion in the fixative much beyond 2 hours will cause swelling.

This chrome-alum fixative was devised in an effort to prepare a fluid that would fix yolk-rich amphibian embryos without shrinkage or hardening. Modifications of standard fixatives and trials of different dehydrating and clearing media did not give sufficiently uniform results to satisfy the requirements of mass production of serial sections for classroom work. It was felt that a suitable fixative for this type of material should contain only the necessary effective amounts of killing and fixing chemicals and that excesses should be avoided. Chrome-alum was selected as a hardener which, it was hoped, would not produce brittleness, since it is commonly employed in treating gelatin photographic films so that they may be sponged without harm while remaining flexible and wet. After its successful use on amphib-

[1]Work performed under the direction of Dr. N. E. Kemp, Zoology Department, University of Michigan.

ian material the fixative was tried on other objects that proved troublesome to prepare in large quantities for classroom use. In each instance the chrome-alum fixative either gave convincing evidence of being superior in producing easily sectioned tissues or disclosed structures not made optically distinct by fixatives such as FAA, Bouin's, Gilson's or Zenker's fluids. Four examples are hereby given to illustrate the use and the properties of the chrome-alum fixative.

Amphibian embryos – Frog embryos or larvae from the gastrula to the 7 mm. tadpole stage were fixed without subsequent crumbling of the yolk in serial sections and with good retention of cytological detail by using the following method: The material was fixed in chrome-alum ½ to 1 hour, rinsed in 70% alcohol, dehydrated through absolute alcohol and cleared in xylene. All times in the alcohols and xylene were kept to the minimum necessary for proper dehydration and clearing. The specimens were embedded in 54° paraffin held at 58 to 60° C. to assure good penetration of the yolk mass. Three changes of paraffin over a period of 3 to 4 hours gave homogeneous infiltration. The yolk sectioned as cleanly as the rest of the animal. Ribbons flattened perfectly and adhered excellently.

Euglena gracilis – Chrome-alum fixative was used on cultures of *Euglena* in an effort to prevent balling up of these protozoa when preparing permanent slides for study of general structure. With chrome-alum not only did the animals retain their natural shape but the flagella, not usually visible in permanent slides, showed plainly, even to the spiral twists on the ends. This technic for preparing permanent slides was then developed: To a quantity of fixative in a centrifuge tube an equal quantity of culture was added; after 1 to 3 minutes fixation, with several shakings, the fixed specimens were rinsed well with distilled water and then stained 1 minute with 1:1 Harris hematoxylin, rinsed in tap water and blued with 70% alcohol plus potassium acetate. They were then passed through the alcohols to 0.5% eosin in 95% alcohol and counterstained 1 minute, rinsed well in absolute alcohol, and then cleared in xylene. The specimens were concentrated and transferred to a small quantity of piccolyte from which they could be readily mounted or stored for future use. All changes of fluid were accomplished by centrifuging at low speed and decanting the supernatant liquid. Mucicarmine also gave an excellent preparation with pale pink flagella and bright red nuclei.

Planaria – Cross sections of Planarians prepared with ordinary fixatives showed marked separations of the tissues. Chrome-alum was tried in an effort to reduce these separations. Though not entirely effective, there was a marked improvement. Even more outstanding, however, was the clear definition of the slime droplets inside the body and extending to the ectodermal border of the animal. These droplets stained brilliant red with eosin and their outlines were

sharp and distinct, in contrast to the blurred appearance and dull color obtained with other fixatives. The animals retained their natural pigment and did not contract unduly when killed. They sectioned and flattened easily. The fixative was poured over the specimens as they were stretched out in a beaker containing a very small amount of water. They were allowed to remain in the fixative from ½ to 1 hour.

Insect larvae – Rapid killing and good fixation of insect larvae was achieved by dropping them into chrome-alum fixative and leaving them for ½ to 1 hour after death. There was no bursting of organs or tearing of tissues, as encountered with other fluids. After clearing in xlyene larvae were allowed to remain in 54° paraffin at 65° C. for 2 to 4 days, or until they were absolutely clear. Even the head and mouth parts sectioned well, and there was no evidence of hardened tissues. Staining was sharp and cell detail clear.

In general this chrome-alum fluid fixes cleanly and rapidly without hardening tissues. If tissues are left too long in the fixative they will swell. The fixative may be followed by most stains and gives delicate detail. Because the ingredients have been held to an effective minimum the fixative is not unpleasant to use in quantity or in confined quarters. It keeps well and does not stain or harm the skin. It will display structures that picric acid, mercuric chloride and bichromate fixatives will not and does not call for time-consuming washings and rinsings. Experience indicates that this chrome-alum fixative is well worth adding to the tools of the microtechnician.

SPECTROPHOTOMETRIC CHARACTERISTICS AND ASSAY OF BIOLOGICAL STAINS

III. THE XANTHENES

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Received for publication April 11, 1950

ABSTRACT.—**In this continuation paper of the work on the chemical and spectrophotometic characteristics of commercial stains, data on the xanthene dyes are presented. In the xanthene group of dyes, it has been found possible to assay pyronin B, eosins B and Y and ethyl eosin by spectrophotometric means. Phloxine B, rose Bengal, and erythrosin B are assayed by the color acid precipitation method. Typical absorption curves are given for these dyes as well as representative spectral and assay data.**

This paper is a continuation in a series aimed at providing revised procedures for the spectrophotometric identification and assay of commercial dyes. In the first paper of the series (Stotz and Conn,

TABLE 1. SPECTRAL AND ASSAY DATA FOR PYRONIN B

Sample	Peak mμ	$\left(\frac{P-15}{P+15}\right)$	D-peak	Assay* per cent
CP–3	554	1.53	0.407	23.4
CP–6	555	1.49	0.710	40.8
NP–3	554	1.61	0.574	33.0
NP–11	555	1.59	0.453	26.0
NP–13	555	1.49	0.533	30.6
NP–14	555	1.60	0.707	40.6

*Calculated by use of absorption coefficient = 3.48 (See Text).

1950) general methods for the spectrophotometric examination and titanous chloride assay were given, and details of these procedures will not be given in later papers. In the second paper (Knapp, Emery and Stotz, 1950) a study of the thiazin series of dyes was presented, and the following pages summarize our study of the xanthene dyes.

PYRONIN B

Holmes and Peterson (1930) have shown that chemical methods for the assay of the pyronins are unsatisfactory, and provided an absorption coefficient to assay these dyes by spectrophotometric means. Since these investigators used a visual spectrophotometer in contrast to our use of the Beckman photoelectric spectrophotometer, it seemed necessary to redetermine the absorption coefficient using a dye sample which had been carefully assayed by Holmes and Peterson. The new value of the absorption coefficient was found to be 3.48

which actually is in quite good agreement with the value of 3.68 recorded by the above investigators.

The complete spectrum of a typical pryonin B sample is shown in Fig. 1, and Table 1 illustrates spectral and assay data for a series

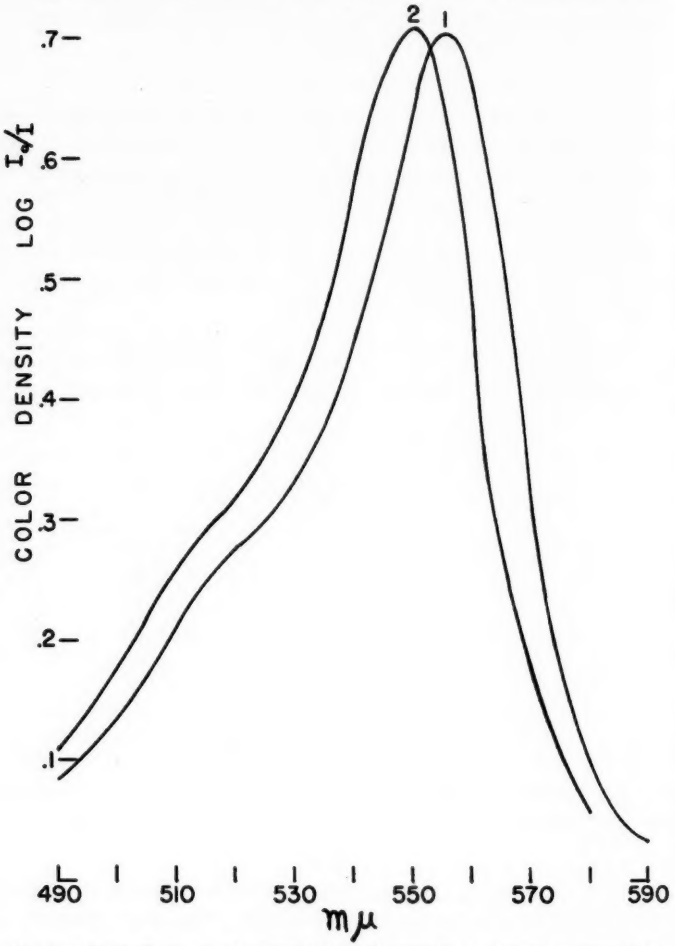

FIG. 1. — Spectral curves of pyronin B and pyronin Y. Curve 1, pyronin B; 2, pyronin Y.

of approved samples. Beer's law of dilution was found to hold at the range of dye concentrations recommended below for spectrophotometric assay.

Spectral: 50 mg. pyronin B dissolved in 250 ml. of 50% alcohol; dilute 5 ml. of this solution to 200 ml. with 50% alcohol. Read in Beckman. Absorption maximum 554 to 555 mμ; ratio P–15/P+15 from 1.49 to 1.61.

Assay: Spectrophotometric. Per cent dye = D-peak × 57.5. D-peak (color density at peak) measured on dye solution described under "Spectral". (Formula given equivalent to per cent dye = $\frac{\text{D-peak}}{3.48}$ for a solution containing 10 mg. per liter.)

Pyronin Y (G)

This dye has not been manufactured by American companies since 1944 and no samples have therefore been submitted recently for certification. For this reason and because possible new products may have somewhat different characteristics than those now in our possession, no detailed data on this stain will be given in this paper. The spectral curve of a typical sample of pyronin Y as previously manufactured is included in Fig. 1 for purposes of comparison with pyronin B.

Eosin B

This dye has been assayed by precipitation of the color acid followed by drying and weighing, a method which is satisfactory although somewhat tedious. A series of analyses by this method showed a constant relation to the color density measured spectrophotometrically, hence the latter method is now recommended for assay of eosin

Table 2. Spectral and Assay Data for Eosin B

Sample	Peak mμ	$\left(\frac{P-15}{P+15}\right)$	D-peak	Assay* per cent	$\left(\frac{\text{Assay}}{\text{D-peak}}\right)$	90% D
LEb–1	517	1.03	0.398	87.8	221	18
	518	1.10	0.392		224	18
NEb–9	518	1.05	0.356	79.7	224	20.5
	519	1.07	0.356		224	20
NEb–11	518	1.06	0.389	89.1	229	19.5
	518	1.01	0.394		226	20
NEb–12	519	1.04	0.381	88.0	231	19.0
	519	1.00	0.379		232	20
				Average =	226	

*Assay by precipitation of color acid.

B. Beer's law of dilution held over the color density range employed. In the dilute alkaline solutions of eosin B employed for spectrophotometry, positions of maximum absorption were found to vary from 516 to 519 mμ. These data are illustrated in Table 2 and a typical spectral curve is presented in Fig. 2.

In Table 2 are also included data for the "90% D width". This measurement is obtained from the plotted spectrum of the dye by measuring the width of the band in mμ at a color density equal to 90% that of the peak value. The 90% D width has been found useful to distinguish "eosin bluish blend" from eosin B (See eosin bluish blend).

Spectral: 50 mg. eosin B dissolved in 250 ml. distilled water; dilute 5 ml. of this solution and 2 ml. of 1% sodium carbonate to 200 ml. with distilled water. Read in Beckman. Absorption maximum 517 to 519 mμ; ratio P–15/P+15 from 1.00 to 1.10.

Assay: Spectrophotometric. Per cent dye = D-peak × 226. D-peak (color density at peak) measured on dye solution described under "Spectral".

Eosin Y

In comparing the color acid precipitation method with spectrophotometric measurements of eosin Y, it was also found that a constant assay/D-peak ratio value was obtained. As in the case of eosin B, Beer's law was found to hold over the assay range. A very constant position of the peak (517 mμ) in dilute alkaline solution was found. Representative spectral data, including the 90% D width values are

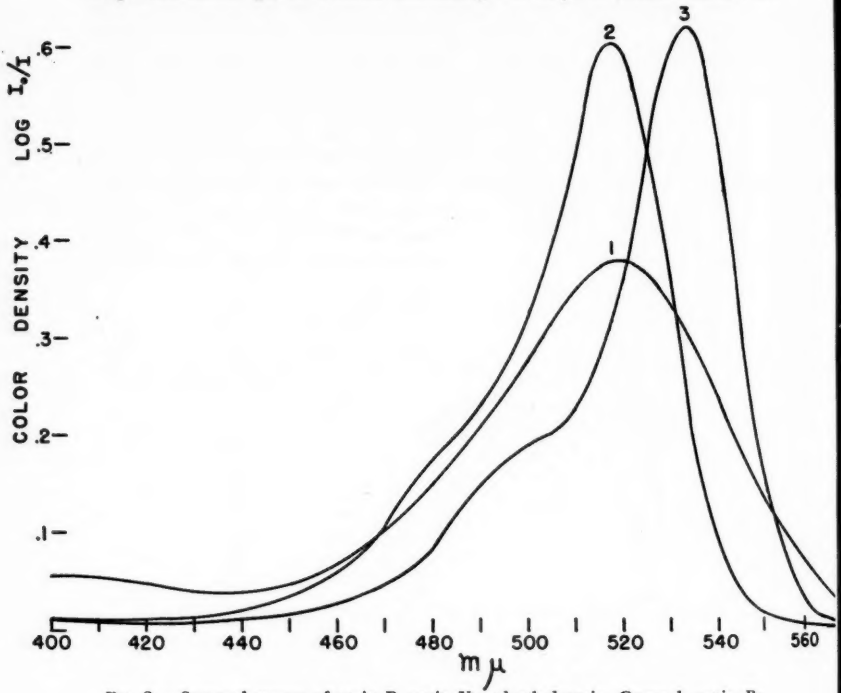

Fig. 2. — Spectral curves of eosin B, eosin Y and ethyl eosin. Curve 1, eosin B; 2, eosin Y; 3, ethyl eosin.

recorded in Table 3. A typical spectral curve for eosin Y is recorded in Fig. 2.

It may be noted that the position of the peak for eosin B and Y is essentially the same, but that the broader absorption range of eosin B can be readily noted in the 90% D width values. It can be noted also that the P−15 : P+15 ratio values for eosin Y are generally larger than those for eosin B.

Spectral: 50 mg. eosin Y dissolved in 250 ml. of distilled water; dilute 5 ml. of this solution and 2 ml. of 1% sodium carbonate to 200 ml. with distilled water. Read in Beckman. Absorption maximum 517 mμ; ratio P–15/P+15 from 1.21 to 1.47.

Assay: Spectrophotometric. Per cent dye = D-peak × 147. D-peak (color density at peak) measured on dye solution described under "Spectral".

Eosin Bluish Blend

This product appears visually to have the color of an eosin B, but spectrophotometric examination reveals it to be principally eosin Y with the addition of a small amount of a red dye. The absorption maximum is at 517 to 518 mμ, and the 90% D width averaged 11 mμ as was true for eosin Y, and in contrast to 19 mμ for eosin B. Consequently samples of "eosin bluish blend" were treated spectrophotometrically and assayed in the same manner as eosin Y.

Table 3. Spectral and Assay Data for Eosin Y

Sample	Peak mμ	$\left(\frac{P-15}{P+15}\right)$	D-peak	Assay* per cent	$\left(\frac{\text{Assay}}{\text{D-peak}}\right)$	90% D
CE–14	517	1.28	0.611	87.5	143	11
CE–15	517	1.21	0.560	81.7	146	11
CE–16	517	1.28	0.586	86.5	148	11
LE–20	517	1.21	0.585	86.6	148	11
LE–21	517	1.39	0.601	89.1	150	11
LE–22	517	1.45	0.602	89.2	150	11
NE–20	517	1.47	0.558	80.7	145	11
NE–21	517	1.40	0.572	82.6	144	11
NE–23	517	1.30	0.591	86.0	146	11
				Average =	147	

*Assay by precipitation of color acid.

Table 4. Spectral and Assay Data for Ethyl Eosin

Sample	Peak mμ	$\left(\frac{P-15}{P+15}\right)$	D-peak	Assay* per cent	$\left(\frac{\text{Assay}}{\text{D-peak}}\right)$
Ec–1.....	531	1.62	0.721	93.2	129
NEc–5.....	532	1.45	0.612	79.1	129
NEc–6.....	532	1.37	0.614	78.7	128
NEc–7.....	532	1.27	0.628	83.1	132
				Average =	130

*Assay by precipitation of color acid.

Ethyl Eosin

A comparison on a series of samples of the color acid precipitation assay with color densities determined spectrophotometrically showed a constant relation. Beer's law of dilution held over the useful color density range. The position of the peak was found to vary slightly, but the majority of the samples showed a peak at 532 mμ in dilute alkaline solution. These data are illustrated in Table 4 and a typical spectral curve for ethyl eosin is presented in Fig. 2.

Spectral: 50 mg. ethyl eosin dissolved in 250 ml. 50% alcohol; dilute 5 ml. of this solution and 2 ml. 1% sodium carbonate to 200 ml. with 50% alcohol. Read in Beckman. Absorption maximum 531 to 532 mμ; ratio P–15/P+15 from 1.27 to 1.62.

Assay: Spectrophotometric. Per cent dye = D-peak × 130. D-peak (color density at peak) measured on dye solution described under "Spectral".

Phloxine B

This dye has previously been assayed by the color acid precipitation method. Although in the series of samples studied, the position of the peak (547 mμ) was quite constant and hence a spectrophotometric assay seemed possible, the assay/D-peak values did not prove to be sufficiently constant among samples to utilize this method. Spectral data for phloxine B are given in Table 5 and a typical spectrophotometric curve is shown in Fig. 3.

Table 5. Spectral and Assay Data for Phloxine B

Sample	Peak mμ	$\left(\frac{P-15}{P+15}\right)$	D-peak	Assay* per cent	$\left(\frac{\text{Assay}}{\text{D-peak}}\right)$
APh–1	547	1.52	0.586	87.0	148
CPh–3	547	1.62	0.550	85.7	156
CPh–4	548	1.57	0.588	87.5	149
CPh–5	547	1.52	0.553	88.5	160
LPh–3	547	1.38	0.594	87.4	147
NPh–9	547	1.42	0.536	80.7	150
NPh–11	547	1.55	0.454	78.5	172
NPh–12	547	1.55	0.504	85.9	170

*Assay by precipitation of color acid.

Table 6. Spectral and Assay Data for Rose Bengal

Sample	Peak mμ	$\left(\frac{P-15}{P+15}\right)$	D-peak	Assay* per cent	$\left(\frac{\text{Assay}}{\text{D-peak}}\right)$
CRb–3	548	1.35	0.390	79.5	204
CRb–4	544	1.22	0.330	75.9	230
LRb–1	544	1.21	0.329	75.5	229
NRb–2	549	1.63	0.375	86.8	231
NRb–4	550	1.61	0.417	88.4	212

*Assay by precipitation of color acid.

Spectral: 50 mg. phloxine B dissolved in 250 ml. of 50% alcohol; dilute 5 ml. of this solution and 2 ml. of 1% sodium carbonate to 200 ml. with 50% alcohol. Read in Beckman. Absorption maximum 547 to 548 mμ; ratio P–15/P+15 from 1.38 to 1.62

Assay: (Color Acid Precipitation Method). Dissolve an accurately weighed sample of about 0.5 g. of dye in approximately 500 ml. of distilled water. Heat to boiling and add slowly with constant stirring, 3 ml. of 6 *N* hydrochloric acid. Cool the hot solution to room temperature and allow to stand at least two hours. Collect the precipitated color acid quantitatively in a weighed Gooch crucible, wash the precipitate with approximately 150 ml. of a 0.2% solution of hydrochloric acid, dry at 110° C. and weigh.

Calculate the dye content of the original sample from the weight of the color acid by means of the following formula:

$$\% \text{ Anhydrous dye} = \frac{\text{Weight of color acid}}{\text{Weight of original dye sample}} \times 105.6$$

(105.6 is the ratio of the molecular weight of phloxine B to that of the color acid, times 100)

Rose Bengal

A comparison of color densities (D-peak) with assays by the color acid precipitation method failed to show sufficiently constant assay/D-peak values to permit adoption of the simpler spectrophotometric type of assay. This may be ascribed in part to variable positions of the peak among the different samples, these occurring between 544

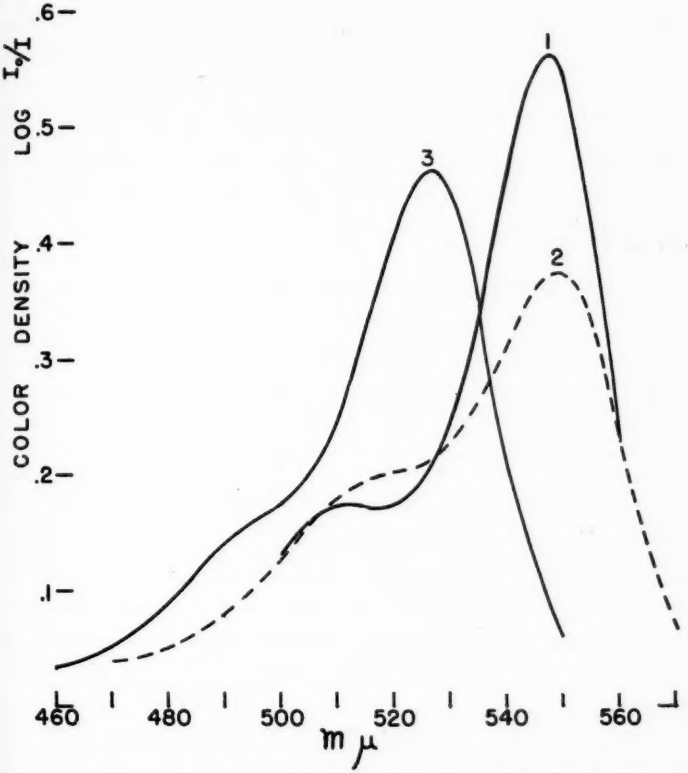

Fig. 3. — Spectral curves of phloxine B, rose Bengal, and erythrosin B. Curve 1, phloxine B; 2, rose Bengal; 3, erythrosin B.

and 550 mμ, although the majority of samples showed a peak at 549 mμ. Preparation of the sample for spectral measurements is described below, and illustrative data are recorded in Table 6. A typical absorption curve for rose Bengal is presented in Fig. 3.

Spectral: 50 mg. rose Bengal dissolved in 250 ml. of distilled water; dilute 5 ml. of this solution and 2 ml. of 1% sodium carbonate to 200 ml. with distilled water. Read in Beckman. Absorption maximum ranges from 544 to 550 mμ; ratio P-15/P+15 from 1.21 to 1.63.

Assay: (Color Acid Precipitation Method). See Procedure under Phloxine B.

Calculate the dye content of the original sample from the weight of the color acid by means of the following formula:

$$\% \text{ Anhydrous dye} = \frac{\text{Weight of color acid}}{\text{Weight of original dye sample}} \times 104.5$$

(104.5 is the ratio of the molecular weight of rose Bengal to that of the color acid, times 100)

Erythrosin B

In attempts to replace the previously used color acid precipitation assay of this dye by a spectrophotometric method, it appeared that dyes of the same manufacture had apparently the same assay/D-peak values, but that dyes of different manufacture showed differences in the ratio (see Table 7). Hence it seemed best to retain the color acid precipitation assay for all samples of erythrosin B rather than attempting to establish spectrophotometric standards for samples of different manufacture. Typical spectral data are shown in Table 7, and an absorption curve in Figure 3.

Table 7. Spetcral and Assay Data for Erythrosin B

Sample	Peak mμ	$\left(\frac{P-15}{P+15}\right)$	D-peak	Assay* per cent	$\left(\frac{\text{Assay}}{\text{D-peak}}\right)$
AEr–1.....	526.5	1.31	0.465	83.5	180
CEr–2.....	525	1.34	0.417	80.3	192
CEr–3.....	525	1.35	0.407	76.7	188
CEr–4.....	525	1.41	0.401	78.7	193
LEr–2.....	525	1.36	0.386	75.1	195
LEr–4.....	524	1.41	0.424	83.5	194
NEr–7.....	526.5	1.25	0.475	85.4	180
NEr–8.....	526.5	1.22	0.483	85.6	177
NEr–9.....	526.5	1.15	0.481	84.5	176

*Assay by precipitation of color acid.

Spectral: 50 mg. erythrosin B dissolved in 250 ml. of distilled water; dilute 5 ml. of this solution and 2 ml. of 1% sodium carbonate to 200 ml. with distilled water. Read in Beckman. Absorption maximum 524 to 526.5 mμ; ratio P–15/P+15 from 1.15 to 1.41.

Assay: (Color Acid Precipitation Method). See Procedure under Phloxine B. Calculate the dye content of the original sample from the weight of the color acid by means of the following formula:

$$\% \text{ Anhydrous dye} = \frac{\text{Weight of color acid}}{\text{Weight of original dye sample}} \times 105.3$$

(105.3 is the ratio of the molecular weight of erythrosin B to that of the color acid, times 100)

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THE CORNEAL EPITHELIUM AS A SOURCE OF MAMMALIAN SOMATIC MITOSES

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Received for publication May 8, 1950

ABSTRACT.—**Dividing cells that occur abundantly in the lowermost layers of the corneal epithelium of various laboratory mammals provide a reliable and readily procurable source of mitotic figures for studies of chromosome number and form. Preparations may be secured quickly by fixing and staining entire eyes in an orcein-alcohol-acetic-acid mixture, or by fixing in acetic-alcohol and then staining with orcein or with leucobasic fuchsin as used in the Feulgen technic. For cytological examination of the epithelial cells and for determination of mitotic rates, the cornea is dissected from the eye and mounted as a flat preparation. Squashes of the epithelial cells are useful if mitotic counts are not required.**

Tissues of the adult mammal have not in general been regarded as favorable for demonstration of the various phases of somatic mitosis. Most studies of chromosome number and morphology in diploid cells of adult animals have been made on spermatogonia prepared for observation by sectioning and staining of the testes (e.g., Painter, 1924). Because the frequency with which dividing cells are encountered in histological preparations of various body tissues is usually very low, efforts have been made to stimulate mitosis experimentally. Stimulation has been effected in mice in hair follicles by shaving the abdomen (Hance, 1927), and in liver cells by partial hepatectomy (Brues and Marble, 1937), by injection of liver homogenate (Wilson and Leduc, 1947) and by intravenous administration of isolated nuclei or fractions thereof containing chromatin (Marshak and Walker, 1945). Aceto-carmine squash preparations of cells of regenerating rat liver have proved useful for studies of chromosome size, form, and number (Biesele, 1944). Painter (1924) found that dividing cells of the inner surface of the amnion of various small mammals provided material for determination of chromosome numbers; and Chrustschoff and Berlin (1935) suggested that chromosomes might be identified in cultures of human leucocytes. Knowlton, Hempelmann, and Hoffman separated homogeneous layers of epithelium two cells thick from mouse ears fixed in 1% acetic acid, and stained in hematoxylin to determine the mitotic index of untreated and irradiated animals.

Within recent years it has been recognized that the basal layers of the epithelium of the cornea of eyes of various laboratory animals afford a constant and more readily accessible supply of mitotic figures. These tissues can be prepared for study by sectioning in paraffin and staining in hematoxylin (Arey and Covode, 1943). The

substantia propria, however, is extremely refractory to sectioning, so that the overlying layers are often badly distorted in slicing the material. Buschke, Friedenwald, and Fleischmann (1943) removed narrow strips extending across the cornea, stained them in hematoxylin, and flattened them on slides for studies of the frequency of mitoses. Kaufmann, Gay, and Hollaender (1944) used whole mounts of the entire cornea for determining mitotic frequency. Each method has some advantages, but preparations of the entire cornea can be made with such rapidity that the technic should have wide application in securing specimens for demonstrating mitotic figures and for studying experimental modifications of rates of division.

The methods of fixing, staining, and detaching the entire cornea, based on our experience with eyes of various mammals – including the mouse, rat, rabbit, cat, dog, and monkey – and to a lesser extent with those of birds, fishes, and amphibians, were outlined in the earlier publication (Kaufmann, Gay, and Hollaender, 1944). A series of inquiries, directed to us since that time, for more detailed description of our methods has prompted the following presentation of technical procedures.

Methods

Our method in its essentials consists of fixing and staining the entire eye and mounting the cornea in a gum sandarac medium. The procedure is based on the pioneer work of Kornfeld (1922) and Politzer (1925), who used flat preparations of whole corneas for the study of mitosis in salamander larvae, and that of Gurwitsch and Anikin (1928), who applied these methods to corneas of rats and dogs. Although in these whole mounts a thick zone of connective tissue (substantia propria) underlies the epithelium, good resolution of detail in the dividing cells of the basal layers is obtainable with oil immersion objectives if dyes are used that selectively color the nuclear materials. Among the best of these are orcein, especially when used as aceto-orcein, and leucobasic fuchsin as employed in the Feulgen reaction. They afford good differentiation in the mitotic cells of the epithelium, but leave the substantia propria relatively colorless. They have certain limitations in cytological studies, however, since they do not stain the nucleoli or the spindle material.

Of the various mammals studied, the mouse and rat are particularly serviceable because of their low cost, the ease with which enucleation is accomplished, and the frequency of mitotic figures (as many as 690 cells in recognizable stages of division have been counted in one square millimeter of cerneal epithelium in the eye of an adult albino rat). These small rodents are killed quickly without anesthetization by dislocating the cervical vertebrae or crushing the medulla. The eyeball can be removed immediately with the aid of fine instruments, care being taken to avoid scratching or otherwise damaging

the corneal epithelium. A brief rinsing in normal saline solution, to remove any debris that has accumulated during enucleation, precedes transfer to the fixative. In this procedure only a minute or two elapses from the time the animal is first handled until the eye reaches the fixative; this interval is sufficiently short to prevent the mitotic inhibition that can be effected by emotional or physiological disturbances (see e.g., Buschke, Friedenwald, and Fleischmann, 1943).

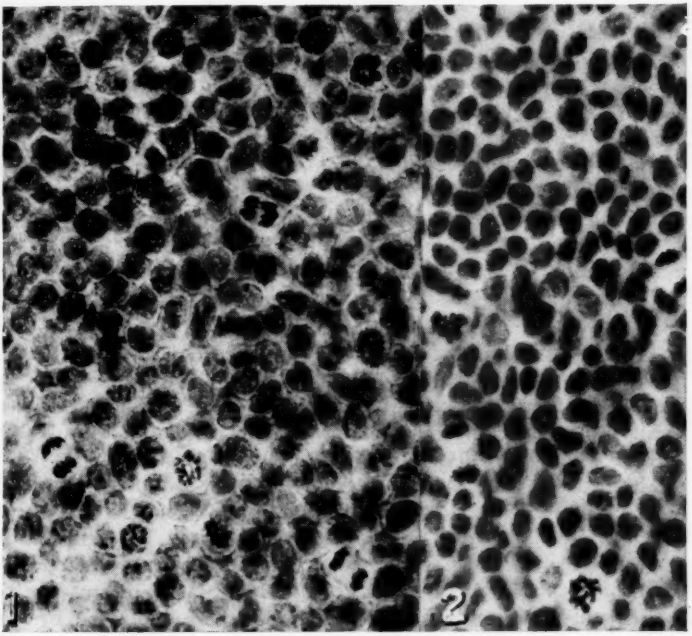

PLATE 1

FIG. 1. — Mouse cornea fixed and stained simultaneously in an orcein-alcohol-acetic acid solution for 30 minutes. ×690.

FIG. 2. — Mouse cornea fixed in acetic-acid-alcohol solution and subsequently stained by the Feulgen procedure. ×690.

Fixing and staining may be carried out as separate and consecutive processes, or a combined fixative-stain may be employed. The latter procedure is slightly less time-consuming than the former, permitting completion of the cerneal preparation in approximately one hour; but the method has certain limitations, as will be indicated later.

Fixation. Satisfactory cytological fixation may be obtained in 30 minutes at room temperature, using acetic-alcohol (3 parts glacial acetic acid and 11 parts 95% ethyl alcohol). If fixation is prolonged,

or material is to be stored in this solution, refrigeration is desirable to prevent the accumulation of excess quantities of ethyl acetate, which seems to interfere with subsequent staining. We have found, however, that eyes kept in acetic-alcohol at 4° C. for four months did not give inferior preparations.

Staining. After fixation, the entire eye may be stained in orcein or by the Feulgen method. In staining with orcein, the eyeball is transferred directly from the fixative to a saturated solution of the dye[1] in 60% glacial acetic acid (prepared by refluxing, cooling, and filtering). Staining continues for a minimum of 30 minutes, after which the eye is transferred to 95% alcohol, where it remains for five minutes. During this destaining interval the cornea is detached from the posterior portion of the eyeball by cutting circumferentially close to the sclerocorneal junction. The dome-shaped cornea is then freed of any adherent conjunctiva, iris, or ciliary body, and four incisions extending toward the center are made from equidistant points on the circumference. (This operation requires from two to two and a half minutes per eye, and, since this is the limiting time factor in the entire schedule, it is possible in a large-scale program for one person to make corneal preparations of the eyes of animals killed at five-minute intervals.) The incisions readily permit flattening of the cornea as it is mounted, with the epithelial surface uppermost, in the gum sandarac medium described by Buchholz (1938) or other similar substances, such as euparal or diaphane, that are miscible with 95% alcohol.

The Feulgen method is an alternative procedure for coloring the chromosomes of the corneal epithelium. After fixation the eye is rinsed in distilled water for 5 minutes, then subjected to the Feulgen procedure as described by Coleman (1938). Satisfactory coloration of chromosomes has been obtained with as little as 20 minutes' staining in leucobasic fuchsin followed by two rinses of 5 minutes each in the SO_2 bath. Longer staining is desirable, however, in studies involving quantitative estimations of chromosomal nucleic acids.

The acidic dyes, fast green and orange G, have been used as counterstains for cytoplasm and nucleoli in these Feulgen-treated preparations. After the SO_2 bath and water rinse, the entire eye is transferred to a 0.25% solution of either fast green FCF or orange G in 95% alcohol. The cells are destained in 95% alcohol while the cornea is being dissected free from the eye.

Simultaneous fixation and staining. The fixative-stain that we have employed is prepared by combining 11 parts of a saturated solution of orcein in 95% ethyl alcohol with 3 parts of a saturated solution of

[1]The orcein used in these studies was a pre-war natural product (Coleman and Bell); 2 g. per 100 ml. — a saturated solution. A sample of synthetic orcein tested for the Stain Commission gave equally good results on corneal squash preparations.

orcein in glacial acetic acid. The entire eye remains in this solution for 30 minutes, and is then destained for 7 minutes in a mixture of 3 parts of glacial acetic and 11 parts of 95% alcohol. During destaining the cornea is detached from the rest of the eye and prepared for flattening. A 3-minute wash in 95% alcohol precedes mounting in gum sandarac. In this procedure the connective tissue of the substantia propria sometimes colors so darkly that it is difficult to secure adequate illumination of the overlying epithelial layers for study with oil-immersion objectives. The addition of a small amount of 1 *N* HCl to the fixative-stain will reduce the intensity of color in these underlying layers, but will also decrease stainability of the chromatin in the epithelial cells. A fixative-stain composed of 2.4 parts of 1 *N* HCl, 11 parts of orcein-saturated 95% alcohol, and 3 parts of orcein-saturated glacial acetic acid, used for 45 minutes to 1 hour, gave lightly colored but clearly defined chromosomes at all mitotic stages.

In the eye of a large animal, the substantia propria zone may be sufficiently thick to warrant some effort to remove a portion of it in order to provide more adequate illumination for oil-immersion studies of the epithelial layers. This operation is best performed while the material is in 30% or 50% alcohol, since in higher alcohols the epithelium tends to wrinkle or shred as it is removed. An incision is made through the epithelium close to the sclerocorneal junction, and the scleral flange is then held with a pair of forceps while the epithelium is gradually loosened from the underlying tissues with a flattened, blunt-tipped probe. With extreme care, separation can be effected along the line of Bowman's membrane, but more frequently the probe passes between somewhat deeper strata of the connective tissue. The detached epithelium should be flattened on a slide and kept covered with a glass slip during subsequent dehydration to prevent the thin sheet of cells from curling. Fluids may be drawn off at one edge of the slip and replaced at the other. To facilitate this process, we cut on the slide, with the aid of a small grinding wheel, a pair of grooves or troughs that will extend within and beyond the two lateral edges of the cover when it is applied.

Squash preparations. If preparations are desired for demonstration of corneal mitoses, but not primarily for determination of rates of division or of chromosome form or number, squashes of the surface epithelium may be made rapidly by adapting a procedure described by Mayer (1892). After enucleation the entire eye is held cornea-downward, one-half inch above the surface of a layer of glacial acetic acid in a small beaker. The vapors of acetic acid fix the surface epithelial cells in a minute or two. The entire eye is then immersed in Ringer's solution (0.14 *M* NaCl) for a few seconds. When the eye is removed from this reagent, it is possible to detach the epithelium

from the underlying tissue with a sharp scalpel. With the blade of the scalpel held perpendicular to the eyeball, the epithelium is scraped from the edge toward the center of the cornea. The loosened epithelium that accumulates in the center of the eyeball spreads out in an intact sheet when the entire eye is returned to Ringer's solution. This layer of cells is then floated onto the surface of a microscope slide while immersed in the saline solution. The slide is removed from the saline and the adhering epithelium is covered with a few drops of aceto-orcein. After 15 minutes a cover-slip is added, and the slide is placed between several layers of filter paper or paper toweling to absorb the excess stain as it is squeezed out by gentle pressure of the thumb or finger applied to the cover. This type of preparation will serve as a temporary mount. A permanent preparation may be made without removing the cover, as described on page 213.

Evaluation of the Methods

Some indication of the adequacy of the corneal preparations for cytological studies is given by the accompanying photographs. Plate 1 shows the abundance of mitotic figures encountered in a typical preparation, and demonstrates the usefulness of such preparations for determining the frequencies of various stages of mitosis (cf. the enlarged photograph of selected stages shown in Plate 2). Reliable counts of chromosome numbers can be obtained from late prophase or prometaphase stages (Plate 2, Fig. 3). Individual chromosomes sometimes reveal the spiral pattern of their chromonemata, as indicated in Fig. 4 of Plate 2. The chromosome that lies to one side of the spindle in this cell bears the same relation to the large group of chromosomes as did the chromosome illustrated by Koller and

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PLATE 2

Various stages of mitosis in cells of corneal epithelia. Fig. 2 and 4 from albino rat; all others from C58 mice. ×1480.

Fig. 1. — Prophase. Fixed in acetic-acid-alcohol and stained by the Feulgen procedure.

Fig. 2. — Late prophase. Fixed in acetic-acid-alcohol, stained with orcein, and counterstained with orange G.

Fig. 3. — Prometaphase. Simultaneously stained and fixed in an orcein-alcohol-acetic-acid solution.

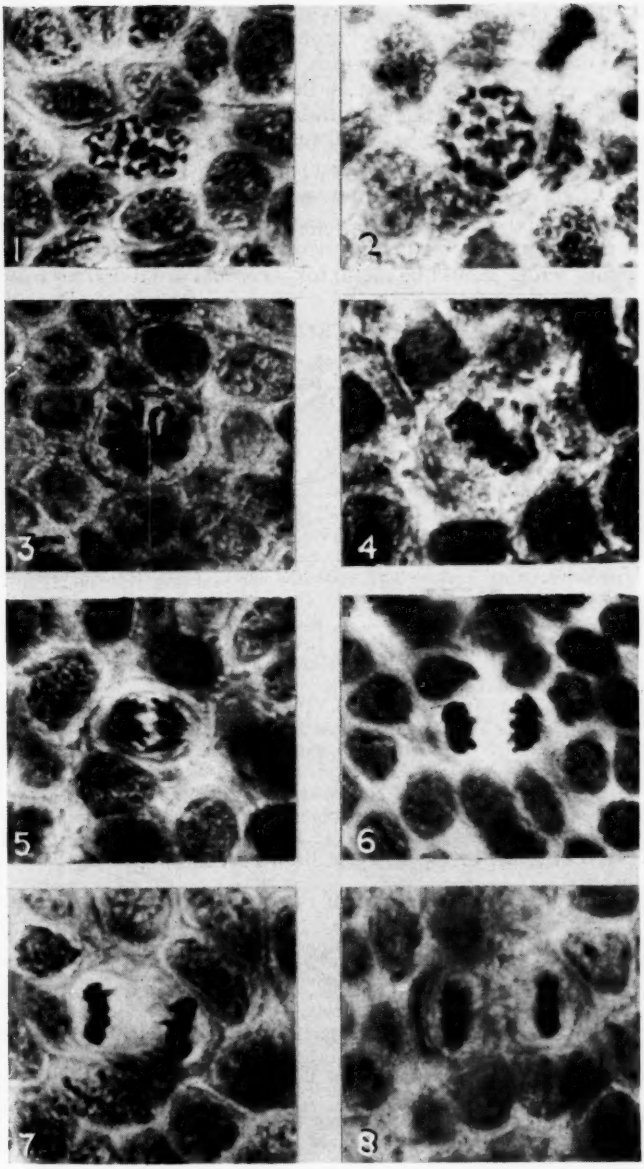
Fig. 4. — Metaphase. Fixed in acetic-acid-alcohol and stained with orcein and orange G.

Fig. 5. — Anaphase. Simultaneously stained and fixed in orcein-alcohol-acetic-acid solution.

Fig. 6. — Late anaphase. Fixed in acetic-acid-alcohol and stained by the Feulgen procedure.

Fig. 7. — Early telophase. Fixed in acetic-acid-alcohol and stained by the Feulgen procedure.

Fig. 8. — Late telophase. Fixed in acetic-acid-alcohol and stained in orcein.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Darlington (1934) in their study of spermatogenesis in the mouse and interpreted by them as the X chromosome.

Because of the ease with which dividing cells can be identified in flat preparations of the cornea, these afford excellent material for studies of the effect on rate of mitosis of various chemicals and different types of radiation. Methods of sampling to insure significant evaluation of experimental as compared with control conditions in this type of study have been discussed previously (Kaufmann, Gay, and Hollaender, 1944). Squash preparations of the corneal epithelial cells provide a rapid method for demonstration of mammalian chromosomes, which should be useful for classroom or laboratory instruction.

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FOOT FOCUSING DEVICE FOR BINOCULAR DISSECTING MICROSCOPE

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Received for publication May 3, 1950

The need is acute for a means of focusing the binocular dissecting microscope in work where both hands are needed for dissection, injection, or other manipulation under high magnification. The simple

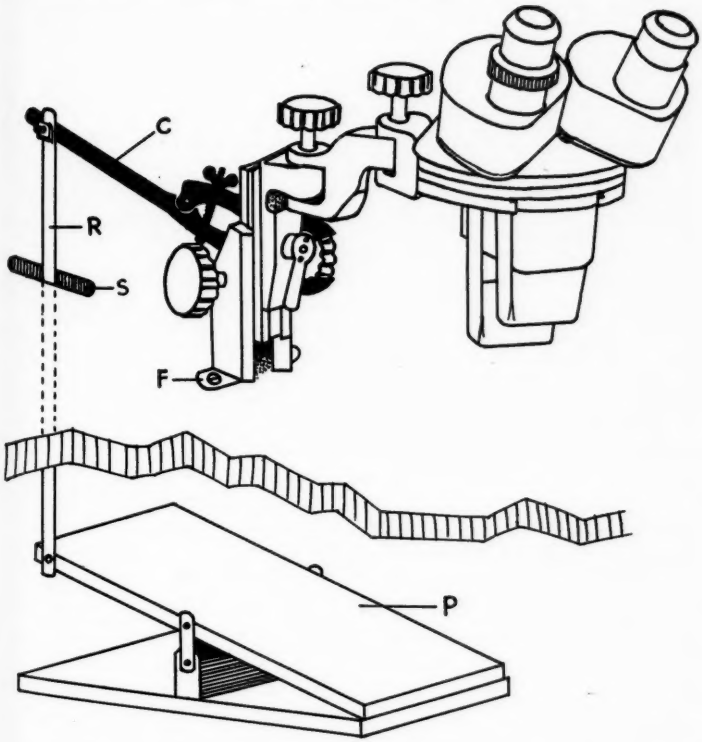

FIG. 1. — A diagram to show the arrangement of microscope and parts of the foot-focusing device. The edge of the table is shown cut away in order to expose the pedal beneath. C, laboratory extension clamp fastened to focusing knob of microscope; F, metal flange which fastens microscope to table top; P, pedal constructed of board hinged in the middle; R, metal rod bolted at upper end to clamp and at lower end screwed to pedal; S, slit in table top through which rod extends.

but very effective device described here was built for studies on *Drosophila* and has been reported in Drosophila Information Service.[1] It has proved so extremely useful that it should be of interest to workers in fields other than *Drosophila* genetics.

The focusing device can be constructed from materials which are available in any laboratory, the cost being almost negligible. The design is shown in Fig. 1 and is much simpler than that described by Stern.[2] Suitable types of microscopes are Bausch and Lomb model RKT–5 and Spencer model 28LG. The binocular should be placed at about a 30° angle from the edge of the table, which arrangement gives plenty of free working space directly in front of the operator. The microscope should be screwed to the table top by means of a a metal flange (F). A laboratory extension clamp (C) is attached to the adjustment knob away from the operator. A metal rod (R) is fastened to the end of the clamp by means of a small bolt. The rod extends through a small slit (S) in the table top to join the pedal (P) which rests on the floor below. In our own case the pedal was made of wood and hinged in the middle. The rod is fastened to the pedal by means of a screw. The pedal base is braced to the table legs by metal strips (not drawn in the figure).

The clamp can be attached or removed from the adjustment knob quickly merely by loosening the thumb screw; thus allowing for a greater range of focus or for hand operation. The device has a direct and positive action with enough leverage for studies under low power, although its need is greatest with high magnifications.

[1]Mickey, G. H. 1949. Drosophila Information Service, No. 23, 105–6.
[2]Stern, Curt. 1936. Drosophila Information Service No. 6. 60.

MOUNTING LARGE SERIES OF SINGLE SECTIONS ON FEW SLIDES

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Received for publication Mar. 17, 1950

This method is applicable to many studies and constitutes a conservation of slides, cover slips, slide filing space and microscopic examination time. Its use here is described in an ecological problem and a problem involving histological examination of 100 rat spleens.

In studying field data on a large series of wild rats, whose reproductive tracts had been preserved previously, it became pertinent to ascertain which males showed presence of sperm and whether this was seasonal. Close to a thousand specimens were involved. It was desirable to mount the sections in a neat compact manner that would result in both a permanent record of each specimen and be sparing of histological materials and storage space.

Tissues had been fixed and stored routinely. Dehydration, paraffin embedding and blocking were also done in the usual manner carrying 50–100 tissues through at a time by using the small gauze bag method (tissue and its serial number in one bag).

Before sectioning and spreading on 2 × 3 inch slides, a glass mounting guide was made by dividing a 48 × 60 mm. area on a 2 × 3 inch slide into squares sufficient to enclose one tissue section and its serial number (Fig. 1). Glass or porcelain ink was used. For spleen sections from rats 30–500 g. body weight and not separated for size, our guide was divided into 24 squares. An ordinary index card was similarly marked off and called the number-key card for entering the tissue serial numbers (Fig. 2).

For the first 24 numbered paraffin blocks to be sectioned and mounted on the one slide, a 2 × 3 inch clean glass slide, prepared with an albumin film in the usual manner, was made ready. This was numbered "1" on the left hand of the slide (Fig. 3). The number-key card was likewise numbered "1". As each block was sectioned, its serial number was entered in a square on the number-key card and its paraffin section mounted on the slide in a position identical to the one entered on the card. Holding the slide together with, and on top of, the glass mounting guide planned the spacing. Thus the first section was placed in the upper left hand corner of the portion of the slide to be covered by the cover glass. The second section from the second block was placed in the square to the right of the first, and so on. Following completion of the first slide of 24 sections, a second one numbered "2" with its accompanying number-key card "2" was similarly prepared. To mount our series of 100

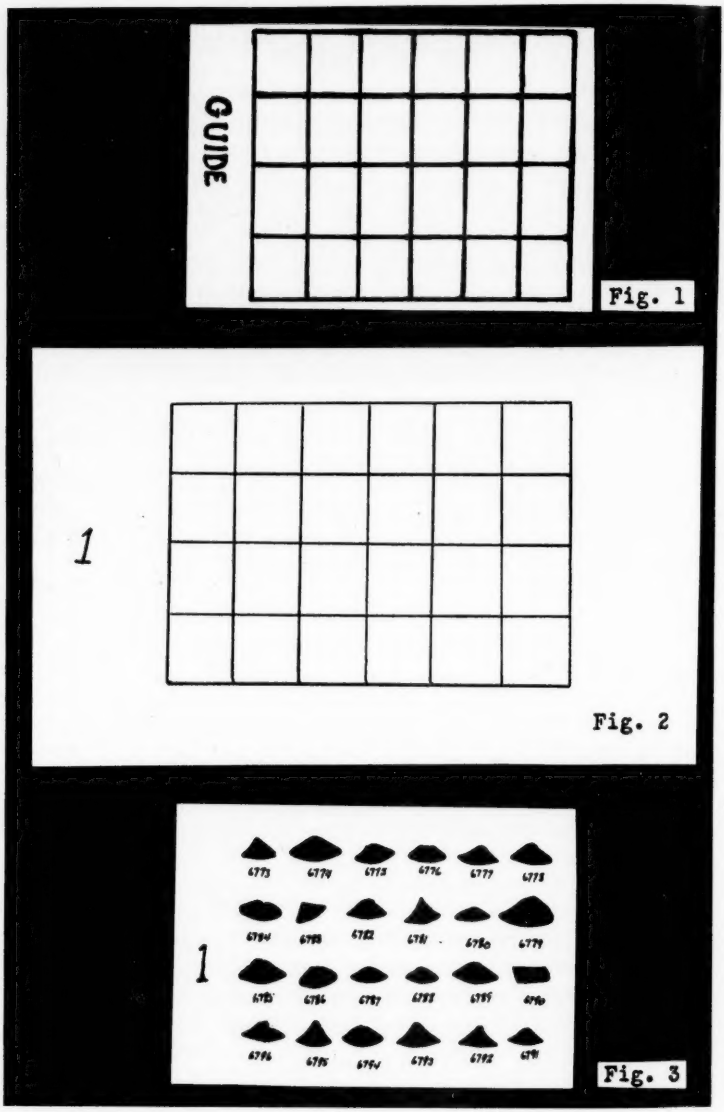

FIG. 1. — Glass Guide

FIG. 2. — Number-key Card

FIG. 3. — Finished Histological Slide

spleen sections from rats of all sizes five 2 × 3 inch slides and five 48 × 60 mm cover glasses were necessary. This constituted a considerable monetary saving in histological slides and cover glass.

Celloidin coating of the slides before staining may be advantageous though we have not had occasion to employ this step as we have never encountered difficulty with sections detaching in staining. After the sections were stained routinely and before mounting in balsam the serial number belonging to each section was penned in India ink on fine tracing paper, trimmed and placed in a position underneath its section. It was held in place temporarily by a very minute amount (size of a pin head or smaller) of balsam. With all numbers placed, the usual amount of balsam was used to finally mount the whole slide with 24 individual sections and their 24 serial numbers under the one cover glass. If too much balsam is used the number slips may float out of place. They may be piloted back to their positions with proper manipulation of balsam currents by applying cautious pressure on top of the cover glass at appropriate points. The slides should then be left in a horizontal position until the balsam has set.

This method of mounting has been used extensively in our examination of several types of tissue, studying one tissue at a time. Staining was uniform if the tissues were fixed and sectioned uniformly, and indeed staining 24 different sections on the one slide simulates the performance of the autotechnicon.

Besides being economic of materials and storage space, histological examination is greatly facilitated since it necessitates the handling of one slide rather than two dozen.

DECREASING THE TIME REQUIRED FOR MAKING AN ALIZARIN SKELETON PREPARATION

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Received for publication Mar. 25, 1950

ABSTRACT.—**The modification described here involves fixing the material in 10% formalin for at least a week, macerated in 2% sodium hydroxide in an oven at 38°C, stained in dilute alizarin solution and finally stored in glycerin. The whole process for a specimen up to 3 cm. long requires less than a month.**

One of the disadvantages in making a good alizarin preparation is the long time required for clearing the tissues. The average time indicated by several authors is about six months.

Attempts have been made by the present writer to decrease the time necessary for the process to a minimum. These involved the nature of the fixing fluid and the concentration and temperature of the clearing (macerating) fluid.

It has been found that fixing the material in 95% alcohol, as recommended by Dawson (1926), Lipman (1935), Richmond and Bennett (1938), and Cumley, Crow and Griffin (1939), minimizes the action of the clearing agent. Instead of the alcohol, 10% formalin (after Williams, 1941) proved to be very efficient for the purpose.

As to the concentration and temperature of the clearing fluid it has been found that a 2% (instead of 1%) solution of sodium hydroxide at 38° C. (instead of room temperature) decreases the time necessary for clearing from several months (Gray, 1929) to only two or three weeks for material up to 3 cm. in length. Unlike Charles and Nobach (1944) the writer obtained no advantage when maceration by sodium or potassium hydroxide and staining with alizarin red S were performed simultaneously instead of separately. On the contrary when maceration was carried out first it was possible to stain the bones to the required density.

The process required about a month.

The whole procedure is as follows:

1. Specimens which have already been preserved in 10% formalin for at least a week need not be skinned nor decerebrated, but large ones may be eviscerated.
2. Transfer specimens to a 2% aqueous solution of sodium hydroxide in an oven at 38° C. The fluid must be changed once or twice daily, for three to seven days, until the bones become clearly visible through the now jelly-like body tissues.

3. Transfer to a solution of 0.1 g. alizarin red S in 1000 ml. of 2% sodium hydroxide, and leave in the oven for twelve hours.
4. Transfer to 2% aqueous sodium hydroxide, changing the liquid every two or three hours, while still in the oven, for another period of twelve hours.

   Great care should be taken during maceration, otherwise the specimen may fall down to pieces, especially if it is left more than necessary in the oven. The correct time is gained by experience. The final stages of clearing must be carried out outside the oven.
5. Transfer to the following mixtures for two days each:
   (a) 80 ml. 2% sodium hydroxide + 20 ml. glycerin.
   (b) 50 ml. 2% sodium hydroxide + 50 ml. glycerin.
   (c) 20 ml. 2% sodium hydroxide + 80 ml. glycerin.
6. Transfer to and store in pure glycerin. The results obtained by dehydrating in cellosolve and clearing in oil of wintergreen, as recommended by Williams (1941), are not as good as those of storing in pure glycerin.

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A RAPID ONE-STEP MALLORY-HEIDENHAIN STAIN FOR CONNECTIVE TISSUE

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Received for publication May 8, 1950

This paper reports a rapid one-step modification of Mallory's anilin blue collagen stain (Mallory, 1936). Several one-step multicolored stains for vaginal smears have been reported by Shorr (1941) and Papanicolaou (1941, 1942), which are variants of Foot's modification of Masson's trichrome stain (Foot, 1933). However, these stains are used for vaginal smears alone, while the technic discussed here applies to tissue sections.

This procedure has three definite advantages over the original and subsequent modifications of the connective tissue stain (Mallory, 1938, and Lillie, 1948): (1) Only one step is involved. (2) Sufficient and desirable coloration is accomplished in five minutes. (3) The intensity of the color is consistent.

The results appear to be the same as those listed by Mallory (1938): Collagenous fibrils stain intense blue; ground substances of cartilage and bone, mucus, amyloid and certain other hyaline substances stain varying shades of blue; nuclei, fibroglia, myoglia and neuroglia fibrils, axis cylinders and fibrin stain red; nucleoli, red blood corpuscles and myelin stain yellow; elastic fibrils stain pale pink or yellow.

The necessity for adjusting the intensity of the aniline blue and acid fuchsin suggested the possibility of combining the two solutions at a modified ratio. The following mixture has proved quite successful. It is immediately effective, and keeps for several months.

STAINING SOLUTION

To 200 ml. distilled water, add the contents listed below one by one, stirring to dissolve the mixture with each addition:

Phosphotungstic acid crystals (Merck)	1 g.
Orange G (C.I. No. 27)	2 g.
Aniline blue, W. S. (C.I. No. 707)	1 g.
Acid fuchsin (C.I. No. 692)	3 g.

METHOD FOR STAINING

Zenker-formal is the preferred fixative, but Bouins, formalin, and alcohol have been used with success. Embed tissue in paraffin, and cut sections at 6μ.

1) Deparaffinize in xylene, pass through graded alcohols to tap water. (If Zenker-formal is the fixative, treat with iodine and sodium thiosulfate.)

2) Stain sections for 5 minutes in staining solution.
3) Wash in running tap water for 3 to 5 seconds.
4) Dehydrate rapidly through graded alcohols, clear in xylene, and mount in balsam.

CONCLUSION

This technic appears to be an improvement over previous modifications of Mallory's collagen stain in that it is more rapid and consistent, and involves only one step. It is suggested for routine staining because it is simple, and the solutions may be used indefinitely.

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NOTES ON TECHNIC

Chromosome Counts in Apple and Strawberry Aided by Paradichlorobenzene

Meyer's paradichlorobenzene prefixation treatment[1] of root tips for shortening of chromosomes and consequent easier counting in smear preparations was tried with apple and strawberry root tips with the paraffin section method and was found to give excellent results. Chromosome counts from the pretreated material were made with exactness and ease. Root tips were kept in the saturated solution of PDB for 1 to 1½ hours before fixing. Root tips had to be washed in water for ½ to 1 hour, or longer; otherwise staining of sections with crystal violet was not very satisfactory. Excellent staining was obtained by keeping the slides in the stain overnight. After this lengthy staining the slides were washed thoroughly and destained quite leisurely without fear of destaining too much. Details of this technic will appear in a forthcoming issue of the Proceedings of the American Society of Horticultural Science. — Haig Dermen, and D. H. Scott, *Division of Fruit and Vegetable Crops and Diseases, Bureau of Plant Industry, Soils, and Agricultural Engineering, Agricultural Research Administration, U. S. Department of Agriculture, Beltsville, Maryland.*

Thick Sections for Embryology

Examination of conventionally prepared slides for embryology and for topographic anatomy of small animals is made unnecessarily difficult because of the thinness of the sections. Experience in this laboratory has shown that for most purposes sections of human embryos 15 mm. or more in length, embedded in nitrocellulose and cut at 75–150μ, and subsequently stained in one of the regressive hematoxylins or some other good selective stain, are much to be preferred. Sections of this kind give a three-dimensional effect that is a revelation to students who are accustomed to working with the usual 20-μ sections. The various types of epithelium, the arborization of glands, the anlage of liver, lungs, and other evaginations of the digestive tract, hair follicles, kidney primordia, branchial clefts, and the paths of nerves, blood vessels, and muscle tracts are much more easily visualized and reconstructed by using thick sections.

If serial nitrocellulose sections are required, they can be numbered successfully by the methods of Rasmussen (Stain Techn., **15,** 113–14, 1940) or Hamilton (Stain Techn., **16,** 125, 1941) and mounted one or more on a slide according to their size.

[1] Meyer, J. R. 1945. Prefixing with paradichlorobenzene to facilitate chromosome study. Stain Techn. **20,** 121–5.

One difficulty in mounting loose nitrocellulose or celloidin sections, particularly thick ones in which tissues of many different types are present, is that they curl and become brittle upon being cleared in mixtures containing xylene or toluene. This can be overcome entirely by dehydrating in 95% alcohol and then transfering directly to the following clearing mixture: phenol 2 parts, cedarwood oil 2 parts, oil of wintergreen 1 part. Some technicians have used terpineol for this purpose. Excess oil is removed and the sections flattened by rolling with a rubber roller and a piece of fine filter paper. Mounting medium and a cover glass are added. Sections prepared in this way two years ago, stained in regressive alum hematoxylin and triosin (Galigher, A. E., *Microtechnique*, 1934) and mounted in Permount show no signs of fading or of crystallization of phenol.

Nitrocellulose or celloidin sections are unsurpassed for lack of shrinkage and for clarity in staining and should be used whenever possible. For small objects such as chick, amphibian, and small pig or human embryos, serial sections in these media may be considered impractical for routine use because of the additional labor involved. In this case embed in a very soft paraffin and section as thick as possible. Better teaching slides are obtained with less work and at a substantial saving in slides and cover glasses.

Thick sections are often objected to because histological detail may be obscured. But slides for instruction in embryology are very seldom used for histology; emphasis is on topography and organogenesis. Moreover, thick sections, especially those in nitrocellulose or celloidin, often are entirely satisfactory for histological detail under higher magnifications provided only that clear, selective stains are used. The effects of a muddy and diffuse hematoxylin, most bulk stains, or any technic which gives poor differentiation of cellular elements or a general opacity to the tissues are much more pronounced than in the usual thin sections.—RICHARD B. TIBBY and SOL BERNICK, *University of Southern California School of Dentistry, Los Angeles, Calif.*

INDEX TO VOLUME 25

Aceto carmine *(See Carmine, aceto)*
Aceto-orcein, 133, 209, 210, 214
Acid alizarin blue, 115
Acid fuchsin *(See Fuchsin, acid)*
Acid violet, 70, 104, 107, 108
Acridine red, 5
Acriflavine, 105, 107, 183
Adamik, E. R. *(See Endicott, K. M.)*
Albert, S. and Leblond, C. P. Age changes revealed by carbonyl reagents in tissue sections, (abs.), 56
Aldehydes, simple histochemical reaction for, 187
Alizarin red S, 223, 224
Alizarin skeleton preparation, decreasing the time required for making, 223
Amethyst violet, 163, 164
  supravital staining of mitochondria with, 163
Ammerman, Frank. Chrome-alum preparation for delicate and difficult fixations, 197
Amphibian chromosomes, aceto-carmine squash technic for, 127
Anilin blue, W.S., 96, 99, 104, 107, 108, 115, 118, 168, 225
Animal microtechnic *(Abs. section)*, 52, 117, 167
Animal tissue, staining of, 33, 35, 53, 95, 111, 117, 167, 168
Anterior hypophysis, human and animal, differential stain for, 95
Apple and strawberry chromosome counts in, aided by paradichlorobenzene, 227
Arginine histochemical method, improved, 143
Arnason, T. J. and Walker, G. W. R. An irreversible gene-induced plastid mutation, (abs.), 54
Arvy, L. and Gabe, M. Mise en évidence simultanée du fer figuré et de las phosphatase alcaline sur coupe á la paraffine, (abs.), 119
Arzac, J. P. Simple histochemical reaction for aldehydes, 187
Assay and spectrophotometric characteristics of biological stains, 57, 201
Auramine O, 104, 107
Autoradiographs of individual blood cells, making, 13
Azocarmine, 96
Azure A, 6, 7, 57, 62, 66, 67, 68
Azure B, 57, 62, 66–68
Azure C, 57, 62, 66–68
Azure II, 104, 107, 183
Bacteria, staining of, 55, 56, 117, 168, 169, 181
Bacterial cells, dye exchange in, and the theory of staining, 181
Bacterial spore stain, simplified, 153
Baker, J. R. The histochemical recognition of certain guanidine derivatives, (abs.), 119
  A new method for oblique microscopical illumination, (abs.) 116
Banny, Theresa M. and Clark, George. New domestic cresyl echt violet, 195
Bartholomew, J. W. *(See Mittwer, Tod)*
Bartholomew, J. W. and Mittwer, Tod. Mechanism of the Gram reaction: 1. The specificity of the primary dye, 103
  Simplified spore stain, 153
Bartholomew, J. W., Roberts, M. A. and Evans, E. E. Dye exchange in bacterial cells, and the theory of staining, 181
Bartholomew, J. W., Evans, E. E. and Nielson, E. D. The effect of esterification of protein carboxyl groups on the staining of bacterial cells, (abs.) 117
Basic fuchsin *(See Fuchsin, basic)*
Basophilia of tissue proteins increase after oxidation with periodic acid, 73
Baudrimont, Albert *(See Dubreuil, George)*
Bernick, Sol *(See Tibby, Richard B.)*
Bindschedler's green, 104, 107
Bismark brown, 183
Bismark brown Y, 104, 107, 118
Bittner, George. Detection of parasites in human excretions, 168
Blood cells, making of individual autoradiographs of, 13
Blood, microtechnic of, 53
Bohn, G. W. *(See Whitaker, Thomas W.)*
Bone, staining of, 223
Book reviews *(Abs. section)*, 115–116
Boyd, George A. *(See Watts, N. P.)*
Boyd, G. A., Casaret, G. W. and Williams, A. I. Making autoradiographs of individual blood cells, 13
Brecher, George. Phase microscopy in the study of reticulocytes, (abs.), 117
  (See also Endičott, K. M.)

Brenner, Sidney. Supravital staining of mitochondria with amethyst violet, 163
Brilliant green, 103, 104, 107
Brooke, M. M. and Goldman, Morris. Polyvinyl alcohol fixative as a preservative and adhesive for protozoa in dysenteric stools and other liquid materials. (abs.), 116
Brown, R. and Rickless, P. A new method for the study of cell division and cell extension with some preliminary observations on the effect of temperature and of nutrients, (abs.), 54, 55

Campbell, A. C. P. The histological preparation of bone marrow particles, uterine curettings and other small tissue fragments (abs.) 52
Carbol fuchsin *(See Fuchsin, carbol)*
Carmine, aceto, 133–135, 167, 168, 209
Carroll, Benjamin, Use of dyestuffs for determining the activity of proteolytic enzymes, 168
Casarett, G. W. *(See Boyd, G. A.)*
Cason, Jane E. Rapid one-step Mallory-Heidenhain stain for connective tissue, 225
Celestin blue, 98
Charpal, O. *(See Verne, Jean)*
Chromosome study, modification of the Schmuck-Metz whole-mount technic for, 21
Ciordia, Honorico and Johnston, Nettie Cate. Rapid staining of nematodes for taxonomic studies, 114
Clark, George *(See Banny, Theresa M.)*
Clearing agent with acetic-alcohol fixatives, cedarwood oil as a, 112
Coburn, W. H. Aerosol as an aid to bone decalcification, (abs.) 116
Cole, W. V. Staining for the comparative histology of the motor ending, 165
Color photomicrography, 116
Committee on mounting media: Interim report on resinous media, 1
Congo red, 41, 44, 104, 107, 118
Conn, H. J. *(See Stotz, Elmer)*
Connective tissue, a rapid one-step Mallory-Heidenhain stain for, 225
Copolymers of isobutyl methacrylate and styrene for mounting media, preparation of, 87
Coverglass, thickness of, 51, 167
Cresyl echt violet, 195, 196
new domestic, 195
Crowdy, S. H. Observations on apple canker: III. The anatomy of the stem canker, 168
Crysoidin Y, 104, 107, 183
Crystal violet, 39, 40, 70, 103, 104, 105, 106, 107, 118, 170, 171, 172, 177, 181, 182, 183, 184, 185, 189, 227
Cucurbitaceae, smear technic for the, 133
Cytological staining, 21, 52, 127, 133, 167, 168, 209, 227

Davenport, H. A. *(See Lhotka, J. F.)*
Davis, Olive Stull *(See Delez, Arthur L.)*
Decalcification, 116
Deformalinizing technics, comparison of, 27
Degenerating nerve fibers, staining and recognition of, 125
Delicate and difficult fixations, chrome-alum preparation for, 197
Delez, Arthur L. 111
Dempsey, Edward W., Singer, Marcus, and Wislocki, George B. Increased basophilia of tissue proteins after oxidations with periodic acid, 73
Dermen, Haig, and Scott, D. H. Chromosome counts in apple and strawberry, aided by paradichlorobenzene, 227
Difficult and delicate fixations, chrome-alum preparation for, 197
Dissecting microscope, binocular foot focusing device for, 217
Dubreuil, George and Baudrimont, Albert. Manuel Theorique et Pratique d'Histologie. *(book review)*, 115
Dyes and their biological uses *(Abs. section)*, 52, 117
Dyes, physiological action, 117

Eayrs, J. T. Apparatus for fixation and supravital staining of tissues by the perfusion method, 137
Edwards, J. E. and Mallory, G. K. Mallory's phosphotungstic acid hematoxylin stain for alcoholic hyalin, (abs.), 117
Embedding, 121
Embedding box for paraffin, improved, 113
Embryology, thick sections for, 227
Emery Jr., Arthur J. *(See Stotz, Elmer)*
Emery Jr., Arthur J., Hazen, Frances Knapp, and Stotz, Elmer. Spectrophotometric characteristics and assay of biological stains: II. The thiazins, 62. III. The xanthenes, 201
Endicott, K. M., Gilman, T., Brecher, G., Ness, A. T., Clarke, F. A. and Adamik, E. R. A study of histo-

chemical iron using tracer methods, (abs.), 53
Eosin B, 6, 7, 8, 201, 203, 204, 205, 208
Eosin bluish blend, 203, 205
Eosin Y, 7, 8, 53, 75, 93, 96, 105, 107, 112, 118, 123, 198, 201, 204, 205, 208
Epithelium, corneal, as a source of mammalian somatic mitoses, 209
Erythrosin, 35, 38, 117
Erythrosin B, 201, 208
Esselen, William B. Jr. *(See Morse, Roy, E.)*
Ethyl eosin, 201, 205, 208
Ethyl violet, 103, 104, 107
Evans, E. Edward *(See Bartholomew, J. W.)*

Fast green FCF, 7, 69–72, 96, 104, 107, 167, 212
Feulgen stain, 56
Fiber identification, 120
Firminger, Harlan I. Carbowax embedding for obtaining thin tissue sections and study of intracellular lipids, 121
Fixations, delicate and difficult, chrome-alum preparation for, 197
Fixations and supravital staining of tissues by the perfusion method, an apparatus for, 137
Fixing agents, 45, 116, 197
Fixing solutions, H-ion concentration of, 45
Fluorescein, 168
Fluorescence microscopy and photomicrography, application of, to wood tissues, 41
Foot's and HIO_4-Schiff technics, staining similarity of, 129
Foster, L. V. Microscope optics, 167
Fuchsin, acid, 6, 7, 8, 70, 71, 104, 107, 108, 117, 118, 225
Fuchsin, basic, 21, 22, 55, 56, 73, 103, 104, 107, 183, 187, 189, 190, 192, 193, 209, 212
Fuchsin, carbol, 104, 107, 193
Fuller, Dorothea, *(See Mackenzie, Locuel)*
Fungi, staining of, 168

Gabe, M. *(See Arvy, L.)*
Gatenby, J. Bronte, and Mousse, Tohamy, A. A. The Sudan black B technic in cytology, (abs.), 52
Gay, Helen, and Kaufman, Berwind P. Corneal epithelium as a source of mammalian somatic mitoses, 209
Gentian violet, 47, 48, 49, 55
Gifford, Ernest M. Jr. Softening refractory plant material embedded in paraffin, 161
Giliman, T. *(See Endicott, K. M.)*
Glycogen, demonstration of, 118
Goldman, Morris, *(See Brooke, M. M.)*
Gomori, George. Improved histochemical technic for acid phosphatase, 81
Gossypium hairs, modified methods for staining the primordial cells of, 39
Gram reaction, the mechanism of, 103, 169
Gram stain, 55, 56, 103, 169
Greco, Jacqueline P. Refractive indices of currently used mounting media, 11
Groat, Richard A. Preparation of copolymers of isobutyl methacrylate and styrene for mounting media, 87
Grossman, M. I. *(See Wang, K. J.)*
Guanidine, demonstration of, 119

Hall, Octavia. Mounting large series of single sections on few slides, 219
Hanna, G. D. Comments regarding the McArthur inverted microscope, (abs.), 51
Harman, John W. Selective staining of mitochondria, 69
Hayes, E. Russell and Hayes, Nancy P. Improved embedding box for paraffin, 113
Hazen, Frances Knapp *(See Emery, Arthur J. Jr.)*
Heiba, A. S. Modified methods for staining the primordial cells of Gossypium hairs, 39
Hemalum, 7, 8, 98, 99, 127
Hematein, 33, 39
Hematoxylin, 6, 7, 33, 34, 53, 54, 88, 92, 93, 95, 96, 98, 111, 115, 117, 123, 127, 167, 190, 194, 198, 209, 210, 227, 228
and Phloxine, use of oxalic acid in staining with, 111
Henneman, Philip H., Wexler, Hilda, and Westenhaver, Mary M. A comparison of eosin-acetone and phloxine-propylene glycol diluents in eosinophile counts. (abs.), 53
Herbert, Simone *(See Verne, Jean)*
HIO_4-Schiff technics, staining similarity of Foot's and, 129
Histochemical method, improved arginine, 143
Histochemical reaction for aldehydes, 187
Histochemical technic for acid phosphatase, 81
Histochemistry, 53, 117, 120

Histochemistry *(Abs. section)*, 56, 119, 168
Histology, methods *(book review)*, 115
Hoffman's violet, 103, 104, 107, 183
Hortega method after paraffin embedding, staining pineal parenchyma by, 35
Hughes, A. F. W. The technique of ciné-photomicrography of living cells, (abs.), 51
Hypophysis, anterior, human and animal, differential stain for, 95

Illumination for microscope, 116
Indicators, dyes as, 168
Interim report of committee on mounting media: Resinous media, 1
Invertebrate histology, 114
Iodine in the gram stain, function of, 169
Iron absorption by tissue sections, 119
Isobutyl methacrylate and styrene for mounting media, preparation of copolymers of, 87

Janus black I, 163
Janus green B, 104, 107, 163, 164, 183
Janus green G, 163
Jeter, W. S. and Wynne, E. S. Acid fuchsin methylene blue agar: A new differential medium for enteric bacteria. (abs.), 118
Johnston, Nettie Cate *(See Ciordia, Honorico)*

Kallman, Burton J. *(See Mittwer, Tod)*
Kaufman, Berwind P. *(See Gay, Helen)*
Kendall, J. D. The chemistry of photographic sensitizing dyestuffs, (abs.) 117
Knapp, Frances *(See Stotz, Elmer)*
Knothe, H. and Maasen, W. Über eine modifikation der Gramfärbung mit Borsäure als Vorbeize and Pikrinsäure-Sublimat-Natrium-acetat-Wasser-Gemish statt Lugolscher Lösung als Hauptbeize. (abs.), 55

Laboratory Hints from the Literature, 51, 115, 120, 167, 168
Laskey, Alice M. Modification of Mayer's mucihematein technic, 33
Lassek, A. A. and Lunetta, Salvatore. Hydrogen-ion concentration of the primary fixing solutions, 45
Leblond, C. P. *(See Albert, S.)*
Lhotka, J. F. and Davenport, H. A. Staining similarity of Foot's and HIO_4-Schiff technics, 129
Lhotka, J. F. and Vaz Ferreira, A. Comparison of deformalinizing technics, 27
Light green SF yellowish, 69, 70, 75, 76, 96, 104, 107, 187, 190
Lillie, R. D., and Mowry, R. W. Histochemical studies on absorption of iron by tissue sections. (abs.) 119
Lillie, R. D., Windle, W. F., and Zirkle, Conway. Interim report of committee on histologic mounting media: Resinous media, 1
Lopes de Fara, J. Valôr do método Faraco para coloração do bacilo de Hansen em cortes. (abs.) 55
Loveland, R. P. Color photomicrography in the laboratory (abs.) 116

Maasen W. *(See Knothe, H.)*
Mackenzie, Locuel, and Fuller, Dorothea. A new vital stain for malignant cells in vaginal secretions, 167
Macklin, Charles C. Mouse epicytes and their intramural processes, 167
The two types of epithelium of the finest bronchioles of the albino mouse as revealed by supravital silverization, (abs.), 53
Makino, Sajiro, and Momma, Eizi. Observations on the structure of grasshopper chromosomes subjected to a new acetocarmine treatment. (abs.), 167, 168
Malachite green, 5, 56, 70, 103, 104, 107, 118, 153, 154, 177, 182, 183, 186
Mallory, G. K. *(See Edwards, J. E.)*
Mallory-Haidenhain stain for connective tissue, 225
Marts, R. O. Application of fluorescence microscopy and photomicrography to wood tissues, 41
Mayer's mucihematein technic, modification of, 21
Mechanism of the Gram reaction, the: I. The Specificity of the primary dye, 103. II. The function of iodine in the Gram stain, 169
Media, dyes in, 118
Meolin, A. E. The efficient manipulation of the microscope, 167
Methyl green, 104, 107, 118, 165, 166, 183
Methyl-green-pyronin staining technic, triethyl phosphate in the, 165
Methyl violet B, 103, 104, 107, 183
Methylene blue, 57, 62, 63, 75–78, 96, 103, 104, 105, 107, 111, 117, 118, 137, 163, 164, 182, 183, 184
Methylene violet, 57, 62–65, 104, 107

Mickey, George H. Foot focusing device for binocular dissecting microscope, 217
Microörganisms *(Abs. Section)*, 55, 117–119, 168
Microradiography, 53
Microscope and other apparatus *(Abs. section)*, 51, 116, 167
Microscope, binocular dissecting, foot focusing device for, 217
Microscope, inverted, 51
Microscopy, advances in, 116
Microtechnic in General *(Abs. section)*, 116
Mitochondria, selective staining of, 69
with amethyst violet, supravital staining of, 163
Mitoses, corneal epithelium as a source of mammalian somatic, 209
Mittwer, Tod, *(See Bartholomew, James W.)*
Mittwer, Tod, Bartholomew, J. W. and Kallman, Burton J. Mechanism of the Gram reaction. II. The function of iodine in the Gram stain. 169
Modification of the Schmuck-Metz whole-mount technic for chromosome study, 21
Momma, Eizi, *(See Makino, Sajiro)*
Morse, Roy E. and Wsselen, William B. Jr. Triphenyl-tetrazolium chloride as a vital strain for dried baker's yeast. (abs.), 55
Moskowitz, Norman. Use of protein silver for staining protozoa, 17
Motor ending, staining for the comparative histology of, 165
Mounting media, preparation of, copolymers of isobutyl methacrylate and styrene for, 87
refractive indices of, 11
resinous, interim report of committee on, 1
Moussa, Tohamy A. A. *(See Gatenby, J. Bronte)*
Mowry, Robert W. A comparison of technical procedures for the histochemical preservation of alkaline phosphatase, (abs.) 119
(See also Lillie, R. D.)
Mucihematein technic, modification of Mayer's, 33

Nassar, Tamir and Shanklin, W. M. Staining pineal parenchyma by a modified Hortega method after paraffin embedding, 35
Nematodes for taxonomic studies, rapid staining of, 114
Nerve fibers, fine, degenerating, staining and recognition of, 125
Nervous system, peripheral, differentiated silver impregnation of conducting pathways 149
Nervous tissue, staining of, 53, 54, 165
Ness, A. T. *(See Endicott, K. M.)*
Neutral red, 105, 107
New fuchsin, 104, 107, 183
Newcomer, Earl H. *(See Tolles, Grant)*
Nicol, J. A. Colin. The Weigert-Pal method for paraffin embedded material: I. Bulk staining and staining of paraffin sections, (abs.), 53, 54
Nielson, E. D. *(See Bartholomew, J. W.)*
Nigrosin, 105, 107, 118
Nile blue 2B, 117
Nile blue sulfate, 183
Notes on Technic, 111–114, 165–168, 227, 228

Orange G, 95, 96, 104, 107, 112, 167, 187, 190, 198, 212, 214, 225
Orange I, 168
Orcein, 50, 209, 210, 211, 212, 213, 214
Oxalic acid in staining with phloxine and hematoxylin, 111

Pacaud, André. Remarques techniques relative à la recherche histochimique de glycogène, (abs.) 118
Panijel, Jacques. Effects de sels biliaires sur certains complexes protéiques Gram-positifs, (abs.) 56
Paradichlorobenzene, chromosome counts in apple and strawberry aided by, 227
Pathological histology, methods, (book review), 115
Pearce, Anthony G. Everson. Differential stain for the human and animal anterior hypophysis, 95
Perfusion method, apparatus for fixation and supravital staining of tissues by the, 137
Periodic acid, increased basophilia of tissue proteins after oxidation with, 73
Phase difference microscopy, *Schistosoma mansoni* miracidium under, 157
Phase microscopy, 51, 116, 117, 157
Phloxine, 53, 96, 111
Phloxine B, 206, 207, 208
Phloxine and hematoxylin, the use of oxalic acid in staining with, 111
Phosphatase, acid, improved histochemical technic for, 81
Phosphatase, demonstration of, 119
Photomicrography *(Abs. section)*, 51, 52, 116

Photomicrography, application of, to wood tissues and fluorescence microscopy, 41
Picric acid, 40, 55, 71, 96, 118, 167, 168, 171, 174, 177, 178, 188, 190, 199
Pineal parenchyma staining by a modified Hortega method after paraffin embedding, 35
Plant material, dry, preservation of, 17
embedded in paraffin, softening of, 161
Plant microtechnic *(Abs. section)*, 54
Plant tissue, staining of, 39
Plastids, staining of, 54
Popham, Robert E. Cedarwood oil as a clearing agent with acetic-alcohol fixatives, 112
Potty, G. L. A simplified procedure for staining acid-fast bacteria in smears and tissue sections, (abs.), 55, 56
Primordial cells of Gossypium hairs, modified methods for the staining of, 39
Protein silver for staining protozoa, the use of, 17
Proteins, tissue, increased basophilia after oxidation with periodic acid, 73
Protozoa, microtechnic for, 118, 168
use of protein silver for staining of, 17
Pyronin, 165, 166
Pyronin B, 201, 203, 208
Pyronin Y (G), 105, 107, 203, 208

Rawlinson, H. E. The use of an iron stain for the study of alveolar development in the mouse mammary gland, 168
Refractive indices of currently used media, 11
Rhodamine B, 105, 107, 177, 183
Richards, O. W. Some recent advances in microscopy (abs.) 116
Rickless, P. (See Brown R.)
Roberts, M. A. (See Bartholomew, J. W.)
Rosa, Charles G. Triethyl phosphate in the methyl-green-pyronin staining technic, 165
Rose Bengal, 105, 107, 201, 206, 207, 208
Root tips, staining of, 54, 55
Roulet, F. Methoden der pathologischen histologie (book review), 115
Royer, G. L. Chemical microscopy in dyeing the finished (abs.) 120

Safranin O, 6, 7, 38, 54, 61, 71, 105, 117, 153, 154, 155, 168, 169, 177, 181, 182, 183, 184, 186
Sartory, P. K. The effects of cover glass thickness on the spherical corrections of objectives, (abs.), 51
Schmuck-Metz whole-mount technic for chromosome study, modification of, 21
Sectioning, 54
Sections, single, mounting large series of, 219
Sedra, Shokralia N. Decreasing the time required for making an alizarin skeleton preparation, 223
Sensitizing dyes, 117
Shanklin, W. M. *(See Nassar, Tamir)*
Silver impregnation of certain conducting pathways in the peripheral nervous system, 149
Silver, protein, for staining protozoa, the use of, 17
Singer, Marcus, *(See Dempsey, Edward W.)*
Skeleton preparation, alizarin, decreasing the time required for making, 223
Sloviter, H. A. Halogenated benzophenoxazine dyes, (abs.) 117
Smyth, J. D. A quantitative study of the osmium impregnation of the contractile vacuole of *Chilomonas parmecium*, (abs.) 118
Spectrophotometric characteristics and assay of biological stains: I. General Methods 57. II. The thiazins 62, III. The Xanthenes 201
Spore stain, simplified bacterial, 153
Stotz, Elmer *(See Emery, Arthur J. Jr.)*
Stotz, Elmer, Conn, H. J., Knapp, Frances, and Emery, Arthur J. Spectrophotometric characteristics and assay of biological stains, 57, 62, 201
Strawberry and apple aided by paradichlorobenzene chromosome counts in, 227
Sudan III, 52
Sudan IV, 52
Sudan black B, 52, 96
Supravital staining and fixation of tissues by the perfusion method, an apparatus, 137

Taylor, E. Winfred. The phase-contrast microscope with particular reference to vertical incident illumination. (abs.), 51
Thiazins, spectrophotometric characteristics and assay of, 62
Thiery, G. L'emploi d'artifices photographiques en technique microphotographique: le "bas-relief", la "solarisation". (abs.), 52

Thin tissue sections and study of intracellular lipids, carbowax embedding for obtaining, 121
Thionin, 6, 7, 57, 62–64, 104, 107, 118, 183, 196
Thomas, Lloyd E. Improved arginine histochemical method, 143
Thomas, O. L. Some observations with phase-contrast microscope on the neurones of *Helix aspersa*, (abs.) 116
Tibby, Richard B. and Bernick, Sol. Thick sections for embryology, 227
Ting, Han-Po. Aceto-carmine squash technic for amphibian chromosomes, 127
Tissue fragments, staining of, 52
Tissue washer for microtechnic, a new type of, 23
Tolles, Grant, and Newcomer, Earl H. New type of tissue washer for microtechnic, 23
Toluidine blue O, 57, 65–67, 75, 98, 104, 107, 118, 166, 177, 183
Triosin, 228

Ultraviolet microscopy, 167
Uranin, 168

Vaarma, Antero. Dry preservation of fixed plant material, 47
Vaginal smears, staining of, 167
Vaz Ferreira, A. *(See Lhotka, J. F.)*
Verne, Jean, Herbert, Simone and de Charpal, O. La localization histochimique de la phosphatase alcaline dans les dents en évolution chez la rat, (abs.), 119
Victoria blue B, 104, 107, 183
Victoria blue R, 103, 104, 107
Victoria blue 4R, 104, 107
Vital staining, 55

Walker, G. W. R. *(See Arnason, T. J.)*
Wall, Patrick D. Staining and recognition of fine, degenerating nerve fibers, 125
Wang, K. J. and Grossman, M. I. A simplification technique for the preparation of sections by freezing and drying, (abs.), 54
Washer for tissue, new type of, 23
Watts, N. P. and Boyd, George A. *Schistosoma mansoni* miracidium under phase difference microscopy, 157
Westenhaver, Mary M. *(See Henneman, Philip H.)*
Wexler, Hilda *(See Henneman, Philip H.)*
Whitaker, Thomas W. and Bohn G. W. Smear technic for the Cucurbitaceae, 133
Whiting, Anna R. A modification of the Schmuck-Metz whole-mount technic for chromosome study, 21
Williams, A. I. *(See Boyd, G. A.)*
Windle, W. F. *(See Lillie, R. D.)*
Wislocki, George B. *(See Dempsey, Edward W.)*
Wrete, Martin. Differentiated silver impregnation of certain conducting pathways in the peripheral nervous system, 149
Wright's stain, 13, 15
Wood tissues, application of fluorescence microscopy and photomicrography to, 41
Wynne, E. S. *(See Jeter, W. S.)*

Xanthenes, spectrophotometric characteristics and assay of, 201

Yeast, staining of, 55

Zirkle, Conway, *(See Lillie, R. D.)*